

**Host Cell Factors in HIV-1 Replication Cycle**

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# **Chapter 1**

## **Introduction**



## **From Discovery to Treatment; Three decades of HIV and AIDS**

It has been more than 30 years since Human Immunodeficiency Virus (HIV) was discovered as the causative agent of Acquired Immunodeficiency Syndrome (AIDS) (1, 2). Since the first identification, HIV has caused more than 25 million deaths and infected at least 60 million people. Despite the development of antiretrovirals and increased understanding of viral biology, HIV continues to be a major global public health problem. According to the United Nations AIDS report on the global AIDS epidemic, there are still more than 30 million people living with HIV (3).

HIV primarily infects and destroys CD4 positive T cells and macrophages (1). According to Centers for Disease Control (CDC) criteria, HIV infection reaches the stage of AIDS when CD4 T cell count drops below 200 cells/ $\mu$ L (Normal level ranges from 500 to 1000 cells/ $\mu$ L) (4). In untreated cases this is usually accompanied by opportunistic infections such as toxoplasmosis and Kaposi's Sarcoma (5).

AIDS was first reported in 1981 at the CDC publication *morbidity and mortality weekly report*. The report included a case history of five homosexual men from Los Angeles who were diagnosed with a rare lung infection called *Pneumocystis carinii* pneumonia which was thought to be caused by a cellular immune

dysfunction (6). This report prompted other case reports around the country and the world. Two years later two separate groups isolated a retrovirus related to Human T cell Leukemia Virus (HTLV) from AIDS patients. Even though these groups initially called this virus HTLV-III and LAV (Lymphadenopathy Associated Virus), it was later renamed HIV based on sequencing and phylogeny analysis (1, 2). The original virus that caused the epidemic was later re-classified as HIV-1 after the discovery of a different subtype of HIV (called HIV-2) that is closely related to simian immunodeficiency virus (SIV) found in sooty mangabeys (7). Evolutionary analysis revealed that HIV-1 most likely evolved from chimpanzees infected with SIV and jumped to humans sometime in early 20th century (8).

Despite the optimism for vaccine development soon after the discovery of the virus, to date vaccine trials have not been successful and today no vaccine is available for preventing HIV infection. In contrast, development of several small molecular drugs called antiretrovirals over the years has made HIV a chronic, manageable condition in parts of the world where access to these drugs is fairly easy. The first FDA approved anti HIV drug came in the form of a nucleoside analog reverse transcriptase inhibitor called azidothymidine (AZT) in 1987 (9). While development of AZT was a significant achievement in the fight against AIDS, high mutation rate of the error prone viral reverse transcriptase led to rapid emergence of drug resistant viruses (10). Following testing and approval of multiple different drugs in the mid 90s targeting different activities of the virus, the

most important breakthrough in treatment of HIV and AIDS came in the form of Highly Active Antiretroviral Therapy (HAART). Using combination of three or more antiretrovirals HAART aims to reduce the emergence of drug resistant viral mutants (11, 12). Today with an arsenal of more than 20 antiretrovirals HAART is the standard treatment for managing HIV infection (Table 1-1).

Antiretrovirals against HIV have been mostly successful in turning the infection into a chronic condition when the treatment is available. However even with multiple different classes of FDA approved antiretrovirals that target different parts of the viral replication cycle (Table 1-1), evolution of drug resistance is inevitable especially for a rapidly mutating virus like HIV (1, 13-15). Even with an effective treatment like HAART, keeping viral replication under control requires strict patient adherence that comes with great personal and financial cost. In addition, majority of antiretrovirals only target two HIV proteins; reverse transcriptase and protease. Therefore if cross resistance to the same type of drugs occurs in a patient, treatment options can become very limited (15). All these reasons make identification of new therapeutic targets a high priority in HIV research. An attractive way to target HIV-1 life cycle that may be less sensitive to viral mutagenesis is the direct targeting of host cell factors that are utilized by the virus. For this reason in recent years a substantial effort has been put to identification of "druggable" host cell factors that are involved in HIV-1 replication. First one of these came in the form of an allosteric modulator of one of the co-

receptors of HIV, CCR5, called maraviroc (16). This drug was FDA approved in 2007 and became the first antiretroviral that targets a host protein.

### **Genome of HIV-1**

HIV-1 belongs to the lentivirus (lenti meaning slow, named due to the slow development of the disease) genus of the family retroviridae (17). Members of this family of viruses contain a single stranded positive sense RNA genome that is reverse transcribed by a virally encoded polymerase called reverse transcriptase to double stranded DNA which is subsequently integrated into the host genome. A representative structure of the viral particle is shown in Figure 1-1. Viral double stranded DNA genome is characterized by long terminal repeat (LTR) regions at each end composed of sequence blocks called U3, R and U5 (Figure 1-2). All retroviruses encode three main genes; *gag*, *pol* and *env* that encode polyproteins which are cleaved into subunits by proteases (Figure 1-2). The *gag* gene encodes for the structural subunits of the virus, matrix (MA), capsid (CA) and nucleocapsid (NC). The *pol* gene encodes for the enzymes of the virus, protease (PR), reverse transcriptase (RT) and integrase (IN). The *env* gene encodes for the surface proteins, called gp120 and gp41 that are used for binding and entry into the target cell. For most retroviruses (including HIV-1) due to a translational stop codon at the end of the *gag* gene, expression of *pol* gene requires a ribosomal frameshift which leads to the production of GagPol polyprotein (18). Without this frameshift only Gag polyprotein is expressed. In

addition to these core genes, some retroviruses including HIV-1 also encode accessory proteins which are involved in regulation of viral replication and evasion of the immune system. For HIV-1 these proteins are; vpu (viral protein u) which has roles in downregulating restriction factor Tetherin (19, 20), tat (transactivator of transcription) which is required for viral transcription (21), rev (regulator of viral protein expression) which exports viral mRNA to cytoplasm (22), vif (viral infectivity factor) which counteracts cellular restriction factor APOBEC3G (23), vpr (viral protein r) which has roles in nuclear import of viral cDNA and arrests cells division (24) and nef (negative regulatory factor) which downregulates cell surface expression of the receptor CD4 and MHC-I to avoid detection by the immune system (25).

### **HIV-1 Life Cycle**

For the purposes of this dissertation HIV-1 replication cycle is divided into two phases: The early phase begins with binding and entry of the virus to the host cell and ends with integration of the viral genome to the host chromosome. The late phase starts with expression of viral proteins from the proviral genome and ends with the maturation of viral proteins after the budding of the viral particle. A diagram of the replication cycle of HIV-1 is shown in Figure 1-3.

### *Early phase*

As an enveloped virus, HIV-1 needs to fuse its lipid bilayer with the plasma membrane of the host cells to deliver the components of the viral particle into the host cytoplasm. Subunits of the envelope (Env) glycoprotein mediate viral binding and membrane fusion. Env protein is produced as a precursor in the cell and subsequently cleaved to two subunits; gp120 and gp41 by a furin-like protease in the endoplasmic reticulum (26). The outer subunit gp120 is required for viral binding while the transmembrane subunit gp41 is required for membrane fusion. Binding of gp120 to the cellular receptor CD4 triggers a conformational change that enables interactions with one of the co-receptors; CCR5 or CXCR4 (27-30). This is then followed by fusion and entry. Since cellular entry of HIV-1 is pH independent, for years it has been thought that viral fusion with the cellular membrane happened at the cell surface (31). However recent evidence obtained using single particle tracking and imaging technologies suggests that at least in some cell types viral fusion occurs at the endosomes (32).

Following membrane fusion, contents of the viral particle are released into the cytoplasm. At this stage viral core which is composed of the capsid protein disassembles in a process called uncoating. Despite being a very important step, uncoating is one of the least characterized parts of the HIV-1 life cycle. Currently most of the controversy is centered on the precise timing and the cytoplasmic location of the uncoating process (reviewed in (33-35)). While strong evidence obtained using capsid mutants indicate that uncoating is coupled to reverse

transcription (36-38), recent data from different studies is contradictory on whether reverse transcription process affects the kinetics of uncoating (39-42). Some evidence suggests that uncoating may be required for the host factors involved in the reverse transcription to access the reverse transcription complex (RTC) (43, 44). However whether the complete capsid disassembly is required for this or not is still not clearly established.

Reverse transcription of retroviruses starts with the synthesis of minus strand strong stop sequence which is primed by a cellular tRNA that is packaged into the virus particle. Following a strand transfer event called first jump, minus strand synthesis is completed. The newly formed DNA/RNA hybrid is degraded through the action of viral RNase H. However 3' and central polypurine tracts can resist this degradation and serve as primers for the plus strand synthesis. Following another strand transfer called second jump, plus strand synthesis is completed and the final product of reverse transcription, double stranded DNA, is formed (Figure 1-4). At this point the complex of host and viral proteins that surround the viral DNA is called pre-integration complex (PIC). In some retroviruses such as HIV-1 this complex is actively imported into the nucleus and the viral DNA is integrated into host chromosome through the action of viral integrase (45).

### *Late phase*

Viral DNA that is integrated into the host genome is called provirus. This provirus uses the cellular transcription machinery, specifically RNA polymerase II, to synthesize the viral mRNAs. Some of these are spliced or unspliced and used as a template for translation of viral proteins such as Gag, GagPol and Env and the accessory factors discussed above. Full length unspliced viral RNA is packaged into the newly formed virions. Each virion contains 2 copies of this RNA. The core genes of the virus Gag, GagPol and Env are produced as polyproteins in the host cell. Envelope protein gets post-translationally cleaved by a cellular protease and glycosylated in the endoplasmic reticulum (26). Assembly of Gag and GagPol on the plasma membrane is achieved through the myristoylated N-terminus of Gag which is inserted into the lipid bilayer of the cell membrane (46, 47). Cleavage of Gag and GagPol into the final products is catalyzed by the viral protease which is part of the Pol (Figure 1-5). This process is called maturation and it doesn't start until the virus budding process is initiated (48).

### **Host Cell Factors**

Same as other viruses from various different families, HIV-1 is an obligate intracellular parasite. Since the HIV-1 genome only encodes for 15 proteins, successful completion of the viral life cycle requires complex interactions with various host cell factors at each stage of the replication cycle. Besides the host factors that are used by the virus (known as cofactors), cellular innate immune



system also expresses many proteins to inhibit viral replication. These are collectively called restriction factors. With the publication of several genomewide RNAi screens that examine HIV-1 replication in recent years the number of host cell factors involved in HIV-1 infection has increased substantially (49-51). However the role of most of these candidate genes in HIV-1 infection remain to be confirmed. For this reason in this part of the dissertation I will describe the major host cell factors which have been studied extensively so far in HIV-1 replication cycle.

### *Cofactors*

#### *Viral Entry*

#### *CD4*

The First cofactor of HIV-1 replication to be identified was the main receptor of the virus, CD4. In the initial days of the HIV/AIDS epidemic it became clear that the immunodeficiency in the patients was primarily caused by the depletion of CD4 positive T cells (1, 2). Soon after the discovery of HIV-1 as the causative agent of AIDS selective targeting and killing of the CD4 but not CD8 positive T cells by the virus was shown *in vitro* (52, 53). In addition, monoclonal antibodies against CD4 were demonstrated to inhibit the replication of HIV-1 in cell culture

(27, 54, 55). These observations together with the fact that successful replication of HIV-1 in B cells following transfection of CD4 led to the conclusion that CD4 acts as the receptor of HIV-1 entry (27, 54). CD4 was also the first retroviral receptor to be identified. CD4 belongs to the immunoglobulin superfamily of transmembrane proteins and has an important role in activation of the T cell receptor mediated signaling pathway through antigen presentation by MHCII (56).

#### *CXCR4 and CCR5*

Despite the initial suspicions that CD4 may not be the only receptor for HIV-1 entry, it took about 10 years for the co-receptors of HIV-1 to be identified. Early studies from multiple groups reported isolation of viruses with differential infectivity in different cell types (57, 58). Some isolates that were found in patients who didn't develop AIDS were found to not to be capable of replicating in certain T cell lines while isolates from patients with severe immunodeficiency could easily infect these cells (58). These results led to classification of viral isolates based on their target cell preference as T cell tropic and macrophage tropic. The reason for this difference finally became clear with the identification of co-receptors of HIV-1. First co-receptor was identified using a cell-cell fusion assay between cells that express HIV-1 gp120 and another expressing CD4 and transduced with pools of a cDNA library. Using this assay the chemokine

receptor CXCR4 (initially called fusin) was identified as the co-receptor of HIV-1 (30). Within a couple of months of the publication of this study another chemokine receptor, CCR5, was identified as a co-receptor of HIV-1 by multiple different groups (28, 29). These studies used the previous observation that beta chemokines RANTES, MIP-1alpha and MIP-1beta inhibited infection of macrophage tropic HIV-1 isolates as the starting point and showed that exogenous expression of the common receptor for these chemokines, CCR5, can make CD4 expressing non-permissive cells infectable by macrophage tropic HIV-1 (28, 29). This discovery quickly led to solving of an interesting puzzle in HIV field. Partners of some HIV-1 infected patients had been observed to be resistant to infection by HIV-1 (59, 60). Analysis of peripheral blood mononuclear cells from two such resistant patients revealed a 32 bp deletion in the gene encoding for CCR5 leading to lack of CCR5 on the cell surface (61). This allele is commonly found in northern European populations, and in the homozygous state can render a person resistant to HIV-1 infection (62). In fact an HIV-1 infected person who had developed an unrelated leukemia became virus free following a bone marrow transplant from a donor with the homozygous CCR5 delta 32 allele (63). Due to the success of this approach in effectively curing HIV infection, recent efforts have focused on targeted mutagenesis of CCR5 using site specific endonucleases such as Zinc Finger Nucleases (64, 65).

## *Reverse Transcription and Uncoating*

### *Cyclophilin A*

Cyclophilin A (CypA) is a peptidylprolyl isomerase which was initially discovered as a protein that binds to the immunosuppressive drug cyclosporin A (66). It was later identified as a binding partner of HIV-1 capsid in a yeast two hybrid screen (67). Initially it was suggested that CypA affected HIV-1 replication through its incorporation into virus particle in the producer cells (68). It was later found that CypA plays an important role in the early phase of the particle whether it is found in the producer cells or not (69). CypA binds to the viral capsid involving amino acid residues G89 and P90 and catalyzes the isomerization of the peptide bond between these residues (70, 71). It is not clear what effect does this isomerization have on the viral capsid core. Despite more than 20 years of research precise mechanism of action of CypA in viral replication is unknown. Inhibition of capsid-CypA binding by mutagenesis or through competitive inhibition via cyclosporine A decreases the accumulation of viral reverse transcription products (72, 73). This indicates that CypA impacts early steps in viral replication such as uncoating or reverse transcription. Multiple models for this action have been proposed. While one model suggests that binding of CypA destabilizes the capsid core promoting the uncoating process, another model suggests that CypA may stabilize the core in low concentrations, while help destabilize it in high concentrations (69, 74-76).

### *Other factors*

Another peptidylprolyl isomerase that is implicated in HIV-1 uncoating is Pin1 (Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) (77). Pin1 has recently been shown to be required for HIV-1 uncoating possibly through its interaction with the phosphorylated viral capsid core (78).

Several host factors with roles in HIV-1 reverse transcription process were identified in the last three decades. Examples of this include survival motor neuron interacting protein 1 (also called Gemin2) which is an integrase binding protein that can enhance reverse transcription (79, 80), DNA topoisomerase 1 (TOP1) which interacts directly with NC and increases HIV-1 cDNA synthesis efficiency (81) and tRNA(Lys3) which is packaged into the virions and acts as the primer of reverse transcription (82, 83).

### *Nuclear Import*

#### *TNPO3*

TNPO3 (transportin 3) is a beta karyopherin that mediates nuclear transport of serine arginine rich proteins in the cell in a RanGTP dependent manner (84). The

role of TNPO3 in HIV-1 life cycle was discovered during a yeast two hybrid screen of integrase interacting host factors (85). In the same study it was also demonstrated that depletion of TNPO3 from the cells via RNAi led to a defect in nuclear entry of the PIC. TNPO3 was also found as an HIV-1 cofactor in two of the genomewide RNAi screens performed recently (49, 50). Later studies showed that knockdown of TNPO3 leads to a change in integration sites of HIV-1 provirus (86). Interest in TNPO3 has increased recently when multiple groups demonstrated the importance of capsid in the function of TNPO3 in the HIV-1 life cycle (87-89). Now biochemical evidence exists for capsid binding to TNPO3 (88). Despite all this, the exact mechanism of action of TNPO3 mediated nuclear import of HIV-1 PIC remains to be discovered.

### *Nucleoporins*

Similar to TNPO3 two members of the nucleoporin family were identified in two of the genomewide RNAi screens for HIV-1 host factors; Nup153 and Nup358 (also known as RANBP2) (49, 50). Members of this family form the building blocks of the nuclear pore complex. Nup153 is found on the nuclear side of the pore complex while Nup358 forms the cytoplasmic filaments of the nuclear pore complex (90, 91). Like TNPO3, depletion of these proteins from the cell via RNAi decreases the accumulation of 2LTR circles in the nucleus indicating a defect in the nuclear import of viral PIC (87). Moreover both Nup153 and Nup358 were

found to be interacting with HIV-1 capsid protein (92, 93). A recent study suggests that Nup358 may be used in docking of PIC to the nuclear pore through its interaction with the capsid (94). The location of Nup153 in the nuclear pore suggests that it may be involved in the exit of the PIC from the nuclear pore complex to the nucleus.

### *Integration*

#### LEDGF/p75

Lens Epithelium-derived Growth factor (LEDGF/p75) is transcriptional co-activator that is a member of the HRP (hepatoma-derived growth factor related protein) family (95). It is one of the two proteins encoded by a gene called *PSIP1* (96). The involvement of LEDGF/p75 in HIV-1 life cycle was implicated when it was discovered as an integrase binding partner in a co-immunoprecipitation assay from 293T cells that were engineered to ectopically overexpress HIV-1 integrase (97). Initial loss of function experiments failed to identify the function of LEDGF/p75 in HIV-1 life cycle (98). It was later found that complete or almost complete knockdown of LEDGF/p75 is required to see a substantial enough effect in viral infection due to the fact that intracellular levels of this protein represent only a fraction of the levels required for its role in viral integration (99). Several studies over the years have accumulated evidence for the current model

of the role of LEDGF/p75 in HIV-1 integration. According to this model LEDGF/p75 functions as a molecular tether between HIV-1 integrase and transcriptionally active chromosomal sites directing the proviral integration to these sites (99-101). Some evidence also suggests that loss of this interaction leads to a decrease in the steady state levels of integrase indicating a role of LEDGF/p75 in stability of integrase (98). Since integration of HIV-1 provirus still happens, albeit at low levels, in the complete absence of LEDGF/p75, it is possible that other factors are involved in directing the viral DNA integration into the host chromosome.

### *BAF*

Barrier to autointegration factor (BAF) was discovered as a part of PIC of another retrovirus called MLV (Murine Leukemia Virus) that prevented viral double stranded DNA to integrate into itself (also known as autointegration) (102). Endogenous function of this protein remains unknown. The same mechanism of autointegration prevention was later found in HIV-1 PICs as well (103, 104). According to these studies BAF acts as a dimeric bridge that can bind to and compact viral DNA in a sequence independent manner to prevent autointegration.



## *Transcription and mRNA export*

### *Cyclin T1*

Once integrated into the host chromosome, expression of HIV-1 mRNAs is driven by the 5' LTR. HIV-1 LTR contains a core promoter region with TATA box and multiple Sp1 transcription binding sites as well as an enhancer region slightly upstream of this core promoter which has binding sites for transcription factor NFkB (105, 106). Early studies of HIV-1 transcripts had shown that in the absence of the viral accessory factor Tat only short abortive transcripts are found in the cell while addition of Tat leads to the production of full length viral mRNA (107). This abortive transcription is thought to be caused by a stem loop structure in the viral mRNA called TAR (Trans activating response) (108). This structure is bound by negative elongation factor (NELF) leading to stalling of RNA polymerase II (109). The mechanism of Tat mediated removal of this block became clear when a member of the transcriptional elongation complex (P-TEFb) called Cyclin T1 was identified as a Tat binding partner (21). According to the current model, Tat binds to TAR through its RNA binding domain and recruits P-TEFb to the promoter region through its interaction with Cyclin T1. This leads to phosphorylation of C terminal domain of RNA polymerase II and elongation of viral transcripts.

## *CRM1*

Formation of nuclear export competent mRNPs (messenger ribonucleoprotein particles) requires coordination of a complex series of processing events which are also tightly coupled to transcription. This presents a significant challenge for some viruses such as HIV-1 that produce unspliced, intron containing mRNAs. HIV-1 gets around this block by using one of its accessory factors Rev that enables the export of its unspliced mRNAs. Rev binds the viral mRNA through a region with complex secondary structure called RRE (rev response element) that is found in the *env* gene coding sequence (22, 110). Nuclear export of Rev bound viral mRNA is achieved by the recruitment of the export complex; CRM1 and RanGTP through nuclear export signal found on the Rev protein. This complex is transported through the nuclear pore complex into the cytoplasm. In the cytoplasm Ran GTPase-activating protein 1 hydrolyzes the Ran-associated GTP into GDP which leads to dissociation of Rev and the viral mRNA from the export complex (111-114).

## *Assembly and Budding*

### *TSG101 and the ESCRT Complex*

Studies in early 90s demonstrated that mutations in the p6 protein of HIV-1 that is found at the C terminus of Gag polyprotein lead to a defect at a late stage in

the viral budding process (115). Since HIV-1 is an enveloped virus, budding of the viral particles requires severing and resealing of the connection between the viral membrane and the plasma membrane. The mutations in p6 that were later mapped to the PTAP domain (also called late budding domain) led to an inhibition of the membrane breakage at the end of the budding process and hence accumulation of immature viral particles that are attached to the cell (116). About a decade after the discovery of the importance of p6 in HIV-1 particle budding, one of the cellular components of this process was revealed with the identification of TSG101 (Tumor susceptibility gene 101) as a binding partner of HIV-1 p6 in a yeast two hybrid screen (117). TSG101 is an inactive homologue of ubiquitin conjugating enzymes (E2) which are normally involved in transferring activated ubiquitin to E3 ligases for ubiquitin post-translational modification (118). TSG101 is involved in sorting of endosomal proteins into multivesicular bodies (MVBs) through its interactions with the ESCRT (The endosomal sorting complexes required for transport) machinery (119). In addition to the endosomal protein sorting, ESCRT machinery has been shown to have an important role in the scission of the membranous stalk that connects the two cells at the end of the cell division (120). It is thought that HIV-1 recruits the ESCRT machinery through the interaction of p6 with TSG101 and hijacks the function of this complex in MVB formation to form the membranous curvature at the plasma membrane and exit the cell following membrane scission which is again provided by the ESCRT machinery (reviewed in (121)).

## *Restriction Factors*

### *TRIM5 $\alpha$*

The existence of restriction factors of retrovirus replications were postulated in the late 60s while studying tumor induction in mice by MLV when it was observed that certain isolates of this virus were able to replicate only in certain strains of mice (122). Due to this inhibition, MLV strains were classified according to their ability to infect NIH (N-MLV) or Balb/c (B-MLV) mice (123). The dominant restriction factor, Fv1 (Friend virus susceptibility factor-1) was later found to be acting on the capsid protein of MLV leading to an inhibition after reverse transcription but before integration (124). The gene causing this inhibition was cloned in the mid 90s by positional cloning (125). It is found to be encoding a Gag-like protein of a mouse endogenous retrovirus (125). Even though Fv1 was only found in mice, a similarly dominant and capsid associated restriction of N-MLV was later reported in some human cells. The factor causing this restriction was named Ref1 (restriction factor 1) at the time (126). In addition, another Fv1-like restriction was observed when HIV-1 and SIV were used to infect several cells from different non-human primates (127, 128). For example HIV-1 infection was blocked in rhesus macaque cells while SIV infection was blocked in squirrel monkey cells (128). Similar to Ref1 and Fv-1, this restriction factor (named Lv1) was dominant and sensitivity of viruses could be mapped to the capsid protein

(128, 129). In 2004, TRIM5 $\alpha$  was identified as the gene encoding the HIV-1 restriction factor in rhesus macaque cells by transferring a cDNA library made from rhesus macaque lung fibroblasts to HeLa cells and selecting the cells resistant to infection by HIV-1 but not SIV (130). Not long after the discovery of TRIM5 $\alpha$ , several groups demonstrated that Ref1 and Lv1 were species specific versions of TRIM5 $\alpha$  (131, 132).

TRIM5 $\alpha$  belongs to the TRIM (Tripartite motif) family of proteins which are characterized by the presence of three motifs in each member named RING, B-Box and coiled-coil (133). In addition to these motifs, TRIM5 $\alpha$  also contains a C terminal domain called B30.2 or SPRY. Rapid evolution of some of the residues found in this domain suggested the importance of this domain in the interaction of TRIM5 $\alpha$  with the virus which was later found to be through the capsid protein (134-137). Interestingly in the genome of some non-human primate species such as owl monkeys SPRY domain of TRIM5 is replaced by another capsid binding gene Cyclophilin A. The resulting fusion protein is named TRIM-Cyp (132).

Despite numerous studies conducted to understand how TRIM5 $\alpha$  inhibits retroviral infection, the exact mechanism of restriction is still unknown. Initial evidence suggested a model involving the binding of TRIM5 $\alpha$  to the incoming viral capsid leading to acceleration of the uncoating process through proteasomal degradation and eventually inhibition of reverse transcription. Supporting this model is the E3 ubiquitin ligase activity associated with the RING domain of TRIM5 $\alpha$  (138, 139). However addition of a proteasome inhibitor doesn't relieve

the inhibition of viral infectivity even though it allows the completion of reverse transcription (140). This indicates that neither proteasomal degradation of incoming capsids nor the inhibition of reverse transcription is required for the anti-retroviral activity of TRIM5 $\alpha$ . While it is still not clearly established how TRIM5 $\alpha$  inhibits the post reverse transcription steps, evidence has emerged from recent studies suggesting that TRIM5 $\alpha$  acts as pattern recognition receptor which signals to innate immune pathways in the cell following recognition of the higher order capsid lattice of the virus (141, 142). Interestingly this signaling pathway has been shown for in some of the other human TRIM proteins as well (143).

### *APOBEC3G*

Multiple studies of HIV-1 infections in late 80s and early 90s showed that viruses deficient in one of the accessory factors, Vif, didn't grow in some human T cell lines such as CEM and Hut78 while they were able to replicate efficiently in others such as Cem-SS and SupT1 (144-146). Due to this discrepancy, cells that didn't support infection with Vif deficient HIV-1 were called non-permissive while the ones that supported the infection were called permissive. Initial studies as well as some later ones have also indicated that this effect was in the producer cells since Vif deficient viruses made in permissive cells were able to infect both permissive and non-permissive cells (147, 148). Presence of an inhibitory factor in the non-permissive cells was confirmed by the dominant non-permissive

phenotype of the heterokaryons generated from fusion of permissive and non-permissive cells (147, 148). The restriction factor was later identified using a cDNA subtraction library approach. In this study to increase the chance of finding the gene specific to non-permissive cells authors used two T cell lines; one permissive and the other non-permissive. This analysis led to identification of APOBEC3G (apolipoprotein B mRNA-editing catalytic polypeptide-like 3G) as the restriction factor in the non-permissive cells that is targeted by Vif (23). In the same study it was also found that APOBEC3G is packaged into virions, hence explaining why non-permissive cells were infectable with virus produced in permissive cells (23).

APOBEC3G belongs to a subfamily of cytidine deaminases which get their namesake from the function of APOBEC1 which was the first member of this family to be identified (149). This family is characterized by the presence of one or two Zinc binding deaminase domains in each member. Soon after its identification it was shown that virion packaged APOBEC3G catalyzed the deamination of cytosine to uracil during minus strand synthesis of viral reverse transcription (150). This leads to the accumulation of G to A mutations during plus strand synthesis. This massive hypermutation is thought to be the reason for the inhibition of the replication of Vif deficient viruses.

In addition to HIV-1, APOBEC3G and some members of the APOBEC3 family have been shown to mediate restriction of several other retroviruses ranging from SIV to human foamy virus as well as endogenous retroelements including Alu

and human endogenous retroviruses (151-155). While HIV-1 and some lentiviruses utilize vif to target APOBEC3 proteins for degradation, other retroviruses such as HTLV and MLV seems to exclude encapsidation of these APOBEC proteins (156). Vif mediated degradation of APOBEC3G has been shown to be achieved by adaptor action of vif between APOBEC3G and a E3 ubiquitin ligase complex leading to polyubiquitination and subsequent degradation of APOBEC3G by the 26S proteasome (157). Recent evidence indicates the requirement of a transcription factor called CBF $\beta$  in this process (158).

### *Tetherin*

Similar to vif, early studies of HIV-1 replication demonstrated that there was a substantial decrease in the amount of virus produced in certain cell types in the absence of the accessory protein vpu (159, 160). Electron microscopy of the non-permissive cells infected with vpu deficient viruses showed that the particles were unable to detach from the surface of the cells (160). This phenotype was subsequently shown to be dominant in heterokaryons made from fusions of non-permissive and permissive cells (161). Later it was found that this phenotype was caused by an IFN $\alpha$  (Interferon alpha) inducible factor and addition of HIV-1 vpu counteracted the tethering of viral particles to the cell surface (162, 163). Therefore unlike in the case APOBEC3G, permissive cells can be made non-



permissive by the addition of IFN $\alpha$ . Using the information accumulated on this restriction factor over the years Neil et al. used microarray analysis from several different cell lines to identify candidate genes. Among these IFN $\alpha$  inducible transmembrane protein Tetherin (also called BST-2 or CD317) fulfilled all the criteria and was identified as the restriction factor blocking the release of HIV-1 particles in the absence of vpu (20). It was subsequently shown that in the presence of vpu Tetherin is downregulated from the cell surface (164).

Tetherin is a single pass transmembrane protein which has an N-terminal cytoplasmic domain followed by a transmembrane domain and a C-terminal extracellular domain with a glycosylphosphatidylinositol anchor at the end (165). Due to the presence of double membrane anchors in its topology, Tetherin has been shown to inhibit budding of a variety of other enveloped viruses in addition to retroviruses (166-169). In addition to vpu of HIV-1 and some SIVs other viruses have evolved different mechanisms of counteracting the effects Tetherin. For example, Ebola virus glycoprotein as well as the envelope proteins of HIV-2 and FIV can antagonize antiviral activity of Tetherin (170-172). Mechanism of the downregulation of Tetherin by vpu has gotten clearer over the years. The current model includes recruitment of  $\beta$ TrCP2 ( $\beta$ -transducin repeat-containing protein 2), which is a member of an E3 ubiquitin ligase complex, by vpu following its direct interaction with the cytoplasmic domain of Tetherin. This leads to ubiquitination of Tetherin (non-lysine) which signals to the HRS (hepatocyte growth factor-regulated tyrosine kinase substrate) component of the ESCRT complex leading

to trafficking and degradation of Tetherin in the lysosomal compartment (173-176). In addition to the degradation of Tetherin, other studies have shown that vpu can sequester Tetherin by either trapping it in the Trans Golgi network or blocking its recycling to the cell membrane (177, 178). It is possible that these antagonistic effects of vpu act in concert to counteract the antiviral activity of Tetherin as opposed to being mutually exclusive.

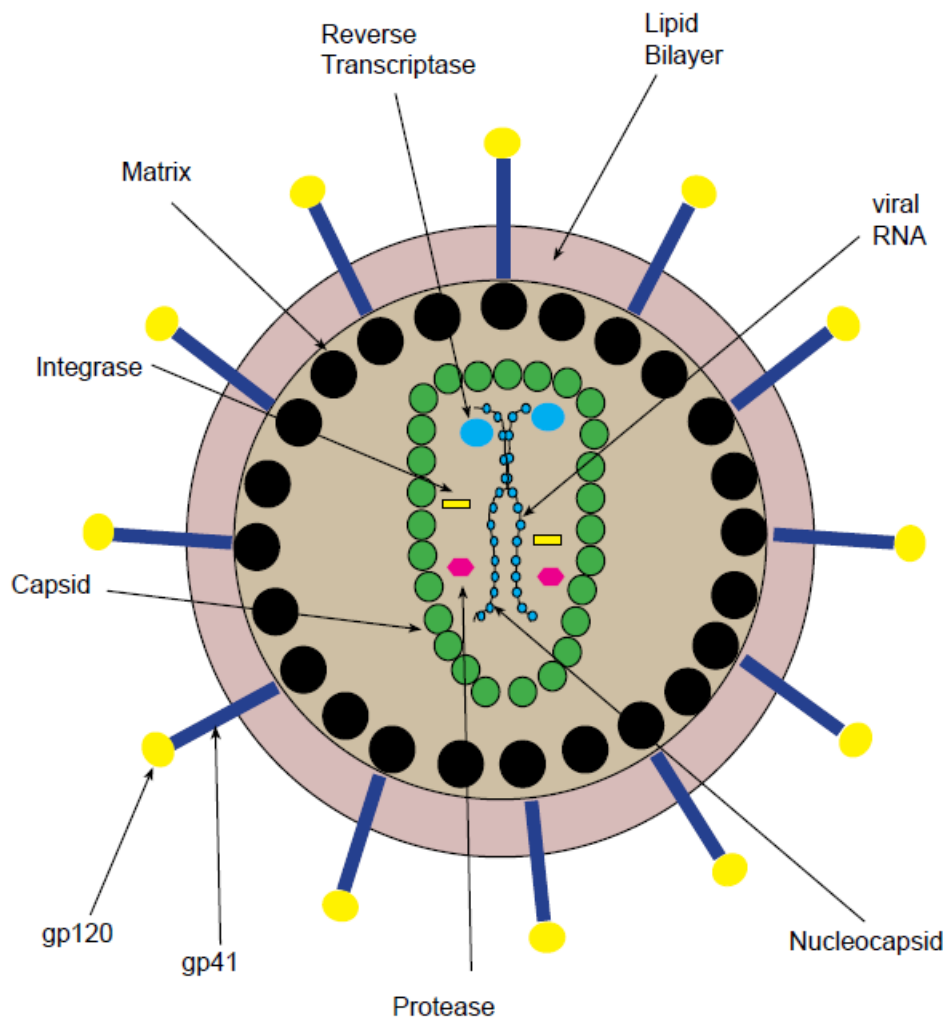
### *SAMHD1*

Low level infectivity of monocyte derived macrophages and dendritic cells by HIV was observed in the early days of HIV research (179). Interestingly this inhibition can be overcome by first infecting the cells with virus like particles containing accessory protein Vpx found in HIV-2 and SIVsm (180, 181). Similar to its counterpart in HIV-1, Vpr, Vpx interacts with the DDB1–CUL4–DCAF E3 ligase complex (182). This interaction was shown to be required for the increase in viral infection observed in myeloid cells after the addition of Vpx (183). Since this E3 ligase complex is involved in ubiquitin mediated proteolytic degradation of targeted proteins, Vpx was suggested to counteract the antiviral activity of a restriction factor by degradation in myeloid cells (180). This factor was later identified as SAMHD1 (SAM domain and HD domain-containing protein 1) using affinity purification of Vpx interacting proteins in non-permissive cells followed by mass spectrometry (184, 185).

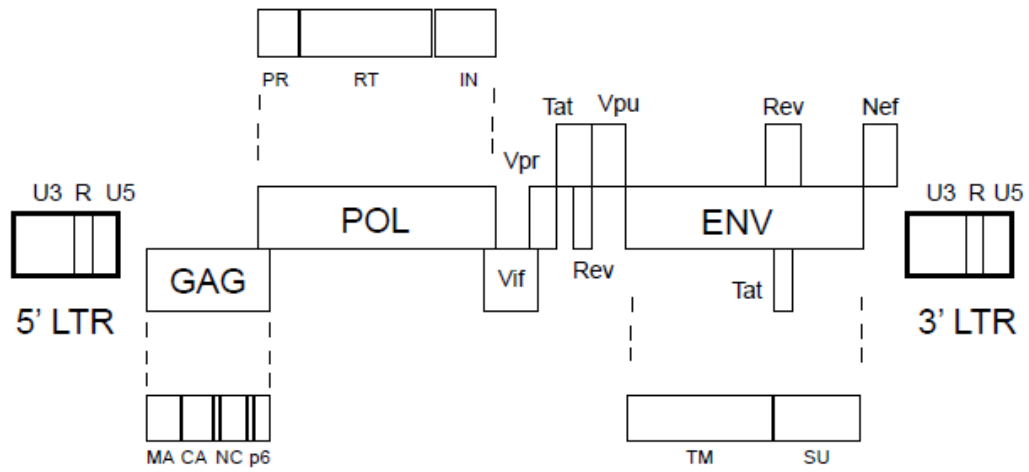
Soon after the identification of SAMHD1 as the HIV-1 restriction factor in myeloid cells, its mechanism of action became clear. SAMHD1 contains a C terminal nucleotide phosphohydrolase domain which suggested that it could have a function in nucleotide metabolism (186). Multiple studies have now shown that SAMHD1 works as a deoxynucleotide triphosphohydrolase (187-189). It restricts replication of HIV-1 by depleting the intracellular dNTP pool. Since non-dividing cells such as monocyte derived macrophages don't require DNA replication, they contain low intracellular dNTP levels. By decreasing the dNTP concentration in these cells SAMHD1 blocks HIV-1 reverse transcription (188). Even though Vpr of HIV-1 cannot antagonize SAMHD1, Vpr from some of the SIV strains can degrade SAMHD1 (190). This suggests that HIV-1 may have evolved to avoid replicating in myeloid cells possibly to escape from an antiviral signal.

Over 30 years of research led to the identification of dozens of host cell factors that have various roles in HIV-1 life cycle as reviewed above. However recent genomewide studies have shown that we still only have a fraction of the whole picture. Hence more in depth studies are required to understand how HIV-1 interacts with the host cell. The goal of this dissertation is to understand the role of host cell factors in the life cycle of HIV-1. I decided to approach this in two parts: In the first two parts I investigated a cellular pathway that was implicated to have a role in retroviral life cycle but never studied in the context of HIV-1

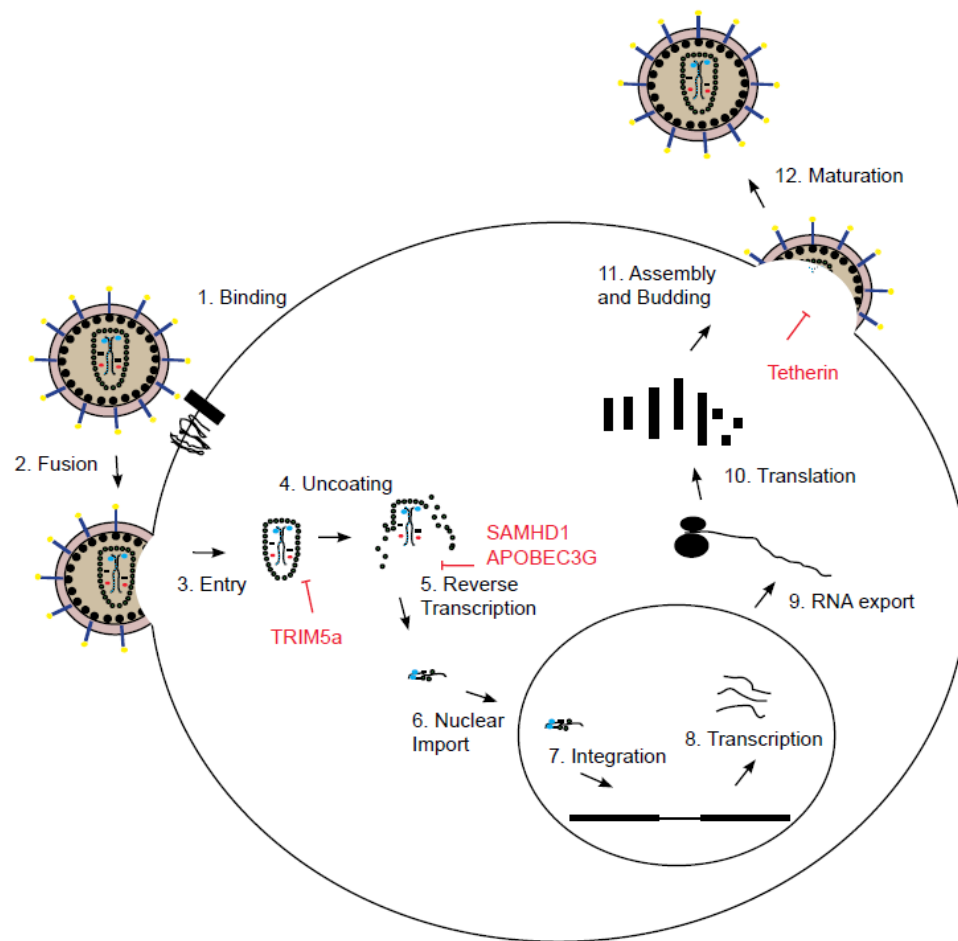
infection. In the last part I studied a block to infection by retroviruses and retroviral vectors in human myeloid cell line.



**Figure 1-1. Schematic representation of HIV-1 viral particle.**

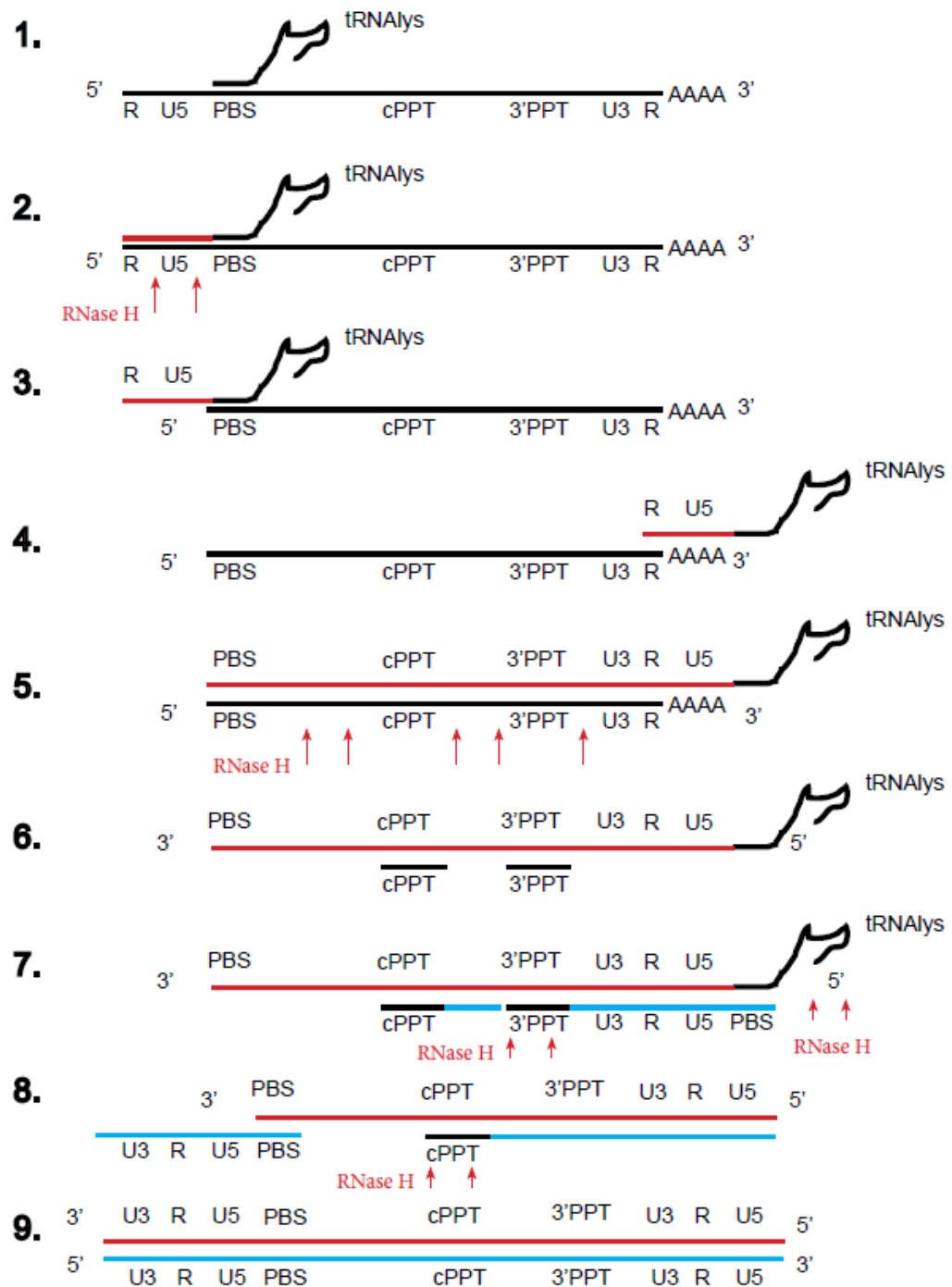


**Figure 1-2. HIV-1 Genome.** Diagram of proviral DNA of HIV-1 with encoded genes.

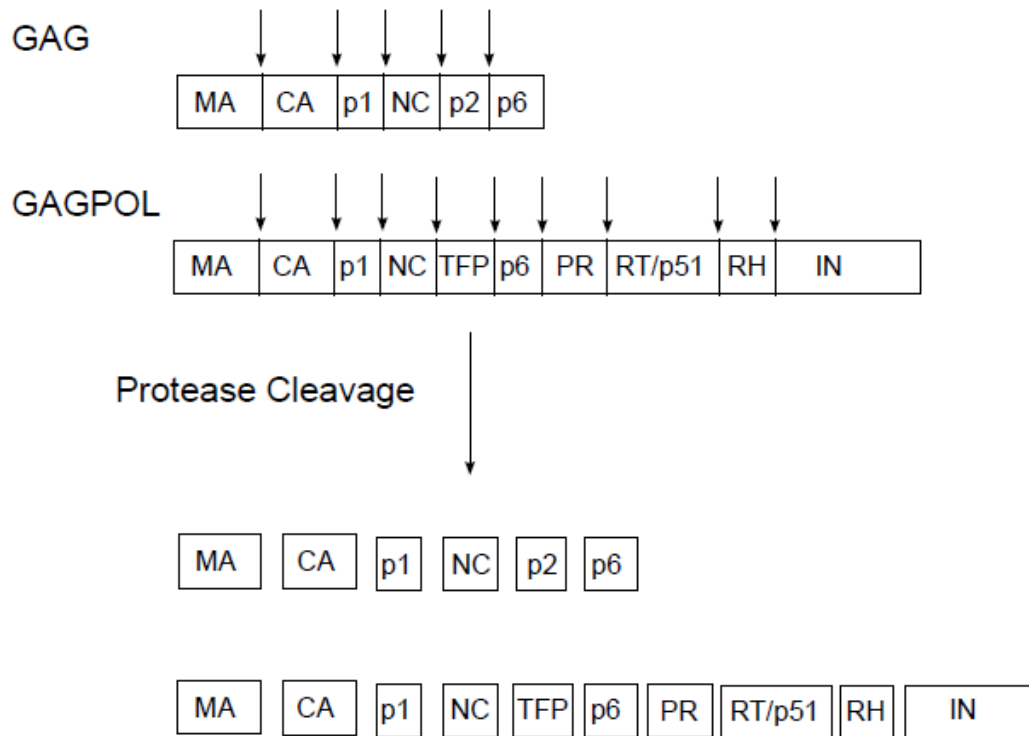


**Figure 1-3. HIV-1 Replication Cycle.** **Step 1.** Binding; HIV-1 particle binds CD4 and one of the co-receptors CCR5 or CXCR4 on the cell surface through surface envelope protein gp120. **Step 2 and 3.** Virion fusion happens with the action of transmembrane protein gp41 and contents of the particle are released into the cytoplasm. **Step 4.** Viral capsid core is disassembled in the uncoating process. **Step 5.** viral RNA genome is reverse transcribed to double stranded DNA with the action of reverse transcriptase. **Step 6.** Pre-Integration complex that contains viral cDNA, several viral and cellular proteins gets imported into the nucleus **Step 7.** Viral double stranded DNA gets integrated into the host chromosome. **Step 8 and 9.** Viral RNAs are transcribed and exported into the cytoplasm. **Step 10.** Viral polyproteins and accessory are produced. **Step 11.** Viral proteins assemble at the plasma membrane and the virus buds out of the cell. **Step 12.** Viral polyproteins are cleaved by the viral protease and the mature particle is formed. Major restriction factors and the part of the life cycle they inhibit are indicated.





**Figure 1-4. HIV-1 reverse transcription process.** **1.** Reverse transcription starts with the binding of tRNA that is packaged into the virus particle at the primer binding site (PBS). **2.** Minus strand synthesis is initiated and continues until it reaches to the 5' end of the viral RNA. This is called strong stop DNA. **3.** RNA strand of this DNA/RNA hybrid is degraded by viral RNase H. **4.** First strand transfer (also called first jump) occurs through the homology of the R region at the LTR. **5.** Minus strand synthesis resumes after the first jump. **6.** RNA strand of the newly formed RNA/DNA hybrid is degraded by RNase H. Two sets of polypurine tracts (PPT) called 3' PPT and central PPT (cPPT) are relatively resistant to this degradation and remain intact. **7.** Plus strand synthesis is primed at the PPTs. Synthesis of plus strand stops after a part of the tRNA is reverse transcribed generating the PBS. **8.** Following RNase H mediated degradation of the tRNA primer and the 3' PPT, second strand transfer occurs (also called second jump) with the annealing of PBS regions from the plus strand and the minus strand. **9.** Synthesis of both strands is completed leading to the formation of the viral cDNA.



**Figure 1-5. Schematic representation of Gag and GagPol processing by HIV-1 protease.** Protease cleavage sites at Gag and GagPol polyprotein are indicated by arrows. Mature forms of viral proteins that make up Gag and GagPol are shown at the bottom of the figure.

Class of Drug	Mode of Action	List of FDA approved drugs
Nucleoside/Nucleotide Analog Reverse Transcriptase Inhibitors	Inhibit viral reverse transcription by competing with the incorporation of nucleotides to the growing viral cDNA chain.	Emtricitabine, lamivudine, zalcitabine, zidovudine, stavudine, Didanosine, Tenofovir, abacavir
Non-Nucleoside Reverse Transcriptase Inhibitors	Inhibit the action of viral reverse transcriptase by directly binding to the enzyme	Rilpivirine, etravirine, delavirdine, efavirenz, nevirapine
Protease Inhibitors	Prevent formation of mature viral particles by directly binding and inhibiting the action viral protease	Amprenavir, tipranavir, indinavir, saquinavir, ritonavir, darunavir, atazanavir sulfate, nelfinavir mesylate
Integrase Inhibitors	Inhibits integration of viral cDNA into the host chromosome by directly binding and inhibiting viral integrase	Raltegravir, dolutegravir
Entry/Fusion Inhibitors	Inhibit viral binding and fusion either by binding to viral gp41 (enfuvirtide) or cellular CCR5 (maraviroc)	Enfuvirtide, maraviroc

**Table 1-1. List of FDA approved antiretrovirals and their mode of action.**

# Chapter 2

## The N-end Rule and Retroviral Infection: No Effect on Integrase

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***Boso G, Tasaki T, Kwon YT, Somia NV. 2013. The N-end rule and retroviral infection: no effect on integrase. Virology Journal 10:233.***

Changes were made for formatting and error correction purposes

### **Authors' contributions**

GB carried out all the experiments, wrote/revised the manuscript and designed all the figures. TT and YTK provided reagents and expertise vital to the study. NVS participated in the design and coordination of the study and helped to draft the manuscript.

## Summary

Integration of double stranded viral DNA is a key step in the retroviral life cycle. Virally encoded enzyme, integrase, plays a central role in this reaction. Mature forms of integrase of several retroviruses (i.e. HIV-1 and MLV) bear conserved destabilizing N-terminal residues of the N-end rule pathway - a ubiquitin dependent proteolytic system in which the N-terminal residue of a protein determines its half life. Substrates of the N-end rule pathway are recognized by E3 ubiquitin ligases called N-recognins. We have previously shown that the inactivation of three of these N-recognins, namely UBR1, UBR2 and UBR4 in mouse embryonic fibroblasts (MEFs) leads to increased stability of ectopically expressed HIV-1 integrase. These findings have prompted us to investigate the involvement of the N-end rule pathway in the HIV-1 life cycle.

Our results showed that infectivity of HIV-1 but not MLV was decreased in N-recognin deficient cells in which three N-recognins (UBR1, UBR2 and UBR4) were depleted. HIV-1 integrase mutants of N-terminal amino acids (coding for stabilizing or destabilizing residues) were severely impaired in their infectivity in both human and mouse cells. Quantitative PCR analysis revealed that this inhibition was mainly caused by a defect in reverse transcription. The decreased infectivity was independent of the N-end rule since cells deficient in N-recognins were equally refractory to infection by the integrase mutants. MLV integrase mutants showed no difference in their infectivity or intravirion processing of integrase.

In conclusion the N-end rule pathway impacts the early phase of the HIV-1 life cycle; however this effect is not the result of the direct action of the N-end rule pathway on the viral integrase. The N-terminal amino acid residue of integrase is highly conserved and cannot be altered without causing a substantial decrease in viral infectivity.

## **Introduction**

Retroviruses are obligate intracellular parasites that must exploit host cell pathways during their life cycle. These activities range from using motor proteins (191), nuclear import (192) and export pathways, to transcription (21) and processing (193) of the viral genome. Studying the role of these cellular pathways in retroviral replication can lead to a deeper understanding of the viral life cycle. This has been highlighted recently with the development of high throughput screening techniques which have implicated numerous proteins that are either required or act to restrict retroviral infection (49-51, 194). One of the processes repeatedly implicated in several genome wide screens is the ubiquitin proteasome pathway that mediates signal dependent degradation of proteins. Different proteins of this pathway have been found in all four genome wide RNAi screens conducted looking for host factors used by HIV-1 (49-51, 194). Although over the years, a variety of studies have implicated the ubiquitin proteasome system in the viral life cycle, research on the role of this essential pathway in retroviral infection has mostly focused on targeting of cellular restriction factors to the proteasome by viral accessory proteins (184, 195, 196). Results of the genomewide studies on host factors suggest that retroviruses may be using the ubiquitin proteasome pathway in the early phase of the viral life cycle.

One of the degradation signals that target cellular proteins to the proteasome is the N-terminal amino acid residue of the protein. First described by Alex



Varshavsky and colleagues in 1986, this N-end rule is an ubiquitin dependent proteolytic system in which the identity of the N-terminal amino acid residue of a protein determines its half life in the cell (197). Even though it was originally discovered while expressing a bacterial protein in yeast cells the N-end rule pathway was subsequently found to be present in all organisms examined including mammals (198), plants (199), and bacteria (200). Hence it is an evolutionarily ancient mechanism for protein degradation. In mammalian cells, amino acids are classified as stabilizing (Met, Ala, Val, Gly, Pro, Ser, Thr) or destabilizing (Glu, Gln, Cys, Asp, Asn, Arg, Lys, His, Leu, Ile, Phe, Trp, Tyr) based on their ability to act as a degradation signal for the N-end rule pathway. Although the N-end rule classifies amino acids as destabilizing for the mammalian cells, it has been difficult to identify the cellular substrates of this pathway. However with the discovery of several signaling components of the N-end rule pathway our understanding of this process has increased in the last decade.

Specificity of the ubiquitin proteasome system is governed by E3 ubiquitin ligases that recognize a degradation signal on the target protein and catalyze the addition of ubiquitin onto the protein. For the N-end rule pathway, these ligases are called N-recognins, and the signal for degradation is termed an N-degron (201). The first N-recognin was discovered in *S. cerevisiae* and termed Ubr1 (201). While Ubr1 is the sole N-recognin in yeast cells, subsequent studies have

identified two homologs of Ubr1 in mammalian genomes, UBR1 and UBR2 (202). These proteins have been shown to have highly similar sequences and overlapping functions (202, 203). Biochemical studies identified two more E3 ligases that can bind to destabilizing N-terminal residues, termed UBR4 and UBR5 (203). These proteins contain a common zinc finger like domain termed a UBR box (203). Mammalian genomes contain three additional genes that code for the UBR box domain, UBR3, UBR6 and UBR7 (203). However these proteins have not been shown to bind any of the destabilizing residues and therefore their role in the N-end rule pathway, if any, is unknown.

While recent studies on N-recognins have substantially increased our understanding of the N-end rule pathway, identification of the cellular substrates of N-end rule has been challenging. Since almost all mammalian proteins are synthesized with an N-terminal methionine, a stabilizing residue, an N-degron can only be created through post translational modifications, such as the removal of the N-terminal methionine (204) or endoproteolytic cleavage. Proteolytic cleavage of certain viral proteins can generate potential substrates for the N-end rule pathway. One viral protein that has been studied with respect to the N-end rule is the integrase of HIV-1. Retroviral integrase is synthesized as a part of a GagPol polyprotein (Pol in spumaretroviruses). Along with other viral proteins, GagPol is packaged into viral particle during assembly and, upon viral budding, the mature form of integrase is generated as a result of a series of proteolytic

cleavage events mediated by the viral protease. We have previously shown that concomitant impairment of three of the N-recognins that bind to destabilizing residues, UBR1, UBR2 and UBR4 in mouse embryonic fibroblasts (MEFs) leads to increased stability of ectopically expressed HIV-1 integrase bearing a destabilizing residue (203). These results and others (205, 206) raised the possibility that HIV-1 (and possibly other retroviruses) may utilize the N-end rule pathway to control the stability of integrase during viral infection.

In this study we investigated the potential role of the N-end rule pathway during the early phase of the life cycle of two different retroviruses; HIV-1 and MLV. Here we show that HIV-1 but not MLV infectivity is decreased in cells where the N-end rule pathway was impaired. We also show that the N-terminal amino acid residue of HIV-1 integrase which has previously been suggested to be a target for degradation by the N-end rule is not targeted by the N-end rule pathway during infection.

## Results

### **Proteolytic cleavage of GagPol generates destabilizing N-end rule residues**

We first examined whether the cleavage of the GagPol polyprotein by HIV-1 protease would generate mature proteins that expose destabilizing N-terminal residues of the N-end rule pathway. Each HIV-1 protease cleavage recognition site in the polyprotein between the subunits differs in its amino acid sequence. Hence the nature of the sequence and its accessibility is thought to determine the specificity for cleavage (207). Furthermore variation among groups or subtypes of HIV may result in sequence variation of the cleavage site. Therefore, we reasoned that if HIV-1 utilizes the N-end rule in its lifecycle, the N-terminal amino acids of mature protein should be conserved as N-degrons across different types, groups and subgroups of HIV-1. The conservation of N-terminal destabilizing residues across other lentiviruses would also suggest a selection pressure to maintain their interaction with N-end rule machinery. A previous study has compared HIV-1 type 1 protease cleavage sites and identified the most common recent common ancestor (MRCA) of protease cleavage sites in HIV-1 for groups M, B and C (208). Of note, 7 proteins are expected to bear N-terminal destabilizing residues. We have further analyzed the conservation of the N-terminal residues of these 7 proteins by comparing the sequences of 1850 HIV-1 isolates from the Los Alamos HIV Sequence Database (<http://www.hiv.lanl.gov/>).

Of the sequences analyzed we have found that 4 proteins (p1, Trans frame octapeptide (TFP), RNaseH and Integrase) have N-terminal amino acid sequences that are conserved relative to the MRCA. The data is summarized in Table 2-1 with respect to the N-terminal amino acid generated and its predicted behavior according to the N-end rule. Result for the cleavage site between Reverse Transcriptase and Integrase is shown in Figure 2-1. In this study we decided to focus on the integrase molecule because it was implicated as a target for the N-end rule (203, 205, 206) and since it is the focus of small molecule development to combat HIV-1.

### **The N-end rule can impact HIV-1 infection.**

We tested if the N-end rule can impact HIV-1 infection. To this end we utilized single cycle HIV-1 reporter vectors and cell lines mutant in N-recognins. We have previously characterized redundant functions of the UBR proteins and hence generated cell lines singly or multiply mutant for UBR proteins (203). These are MEFs from UBR1<sup>-/-</sup>, UBR2<sup>-/-</sup> and UBR1<sup>-/-</sup> UBR2<sup>-/-</sup> strains of mice. We have also previously generated MEF lines that are deficient in all three UBR box proteins (UBR1, UBR2 and UBR4) that have been shown to bind type 2 destabilizing residues by transducing UBR1<sup>-/-</sup> UBR2<sup>-/-</sup> cell lines with 3 different shRNAs against UBR4 mRNA (to account for any off target effects) and a shRNA targeting luciferase mRNA as a control. UBR4 knockdown was verified by

immunoblotting (203). Wild-type MEFs transduced with shRNA to UBR4 mRNA were used to investigate the effect of UBR4 knockdown alone.

We infected N-recognin deficient MEFs with a HIV-1 EGFP vector pseudotyped with VSVG. Infectivity was reported as EGFP expression and was scored by flow cytometry 72hrs after infection. Figure 2-2 illustrates that the infectivity of HIV-1 was unchanged over a range of MOIs (Multiplicity of Infection) comparing wild type (WT), MEFs for UBR1  $-/-$ , UBR2  $-/-$ , UBR4 KD, and the double deletion MEF UBR1  $-/-$  UBR2  $-/-$ . We observed a substantial decrease (5-6 fold) only in cells that are depleted in UBR1, UBR2 and UBR4. We confirmed that this decrease in infectivity is not due to a defect in the reporter or the expression of the reporter by using a different reporter (DS-Red) and a different promoter (CMV) (data not shown).

### **HIV-1 infection is impaired at reverse transcription or uncoating in N-recognin deficient cells.**

These results indicate that N-end rule pathway can impact the early phase of the HIV-1 life cycle. However decrease in infectivity by itself is not enough to determine whether the N-end rule acts on HIV-1 proteins or a cellular protein that is involved in infection. To better pinpoint the stage that is impaired we

determined which step in the viral life cycle is affected in the N-recogin deficient MEFs. Following infection we isolated total DNA from MEFs at different time points and tracked the relative accumulation of early reverse transcription products and 2LTR circles by qPCR. As shown in Figure 2-2 the infection in WT cells compared to UBR1<sup>-/-</sup> UBR2<sup>-/-</sup> cells are similar. Therefore we chose to compare UBR1<sup>-/-</sup> UBR2<sup>-/-</sup> LuciRNAi to UBR1<sup>-/-</sup> UBR2<sup>-/-</sup> UBR4RNAi cells. As shown in Figure 2-3A, UBR1<sup>-/-</sup> UBR2<sup>-/-</sup> UBR4RNAi cells showed around 3-4 fold decrease in the accumulation of first jump products. Moreover the rate of increase in the accumulation of first jump products were found to be lower in the triply deficient cells especially in the early time points (compare 4 hour and 6 hour time points) indicating a defect in reverse transcription. However the first point we analyzed also showed around 2 fold difference in the amount of first jump products between the control and the triply deficient cells, which suggests that another defect before the first jump step of the reverse transcription either at the start of reverse transcription or during the uncoating could contribute to this phenomenon. The difference we observed in the accumulation of reverse transcription products was further enhanced when we looked at the relative production of 2LTR circles. As illustrated in Figure 2-3B, the UBR1<sup>-/-</sup> UBR2<sup>-/-</sup> UBR4RNAi cells exhibited up to 7 fold less 2LTR circles compared to the control cells. However, since the 2LTR circles are only a subset of the reverse transcription products that enter the nucleus we do not observe any defect in nuclear import. Furthermore it has been reported that in the presence of mutant

non-functional integrase 2LTR circles are increased when compared to infection with wild type integrase (209, 210). Hence we compared the ratio of first jump to 2LTR circles at 24 hrs. The data show that for UBR1-/- UBR2-/- UBR4RNAi Luciferase control cells the ratio is  $216.7 \pm 34.6$ , while the ratio in triply mutant cells is  $250.3 \pm 53.9$ . This indicates that the integrase function is not impaired in the triply mutant cells. We conclude that the triply deficient cells are impaired in early reverse transcription or uncoating.

### **The N-end rule does not impact MLV infection.**

Similar to HIV-1, proteins of other retroviruses also harbor N-terminal residues that make them susceptible to the N-end rule. For example mature MLV integrase also bears a type 2 destabilizing residue isoleucine making it a potential substrate for the N-end rule pathway (211). A comparison of different retroviral integrase N-terminal residues revealed that all lentiviruses examined, gammaretroviruses and spumaretroviruses harbor destabilizing residues. However, this is not universal to retroviruses, since alpha, beta, delta and epsilon retroviruses harbor a stabilizing residue (Figure 2-4). To test whether MLV infection is affected by the impairment of the N-end rule pathway, we infected our N-recognin deficient MEFs using a MLV based EGFP vector pseudotyped with VSVG. In contrast to HIV-1, MLV infectivity was only slightly decreased in UBR1-/- UBR2-/- UBR4KD cells compared to the other cells tested (Figure 2-5). Notably



the slight decrease was also present in the control UBR1  $-/-$ , UBR2  $-/-$ , Luciferase RNAi cell line. This leads us to conclude that the impact of the N-end rule on MLV infection is minimal. This also allows us to conclude that the decrease observed for HIV-1 on the triply deficient cells is not due to an entry defect since the MLV and HIV vectors are pseudotyped with VSVG and hence utilize the same mode of entry.

### **The N-terminal residue impacts intravirion stability of integrase.**

The observed differential effect of N-recognin deficiency on HIV-1 and MLV prompted us to question the previous suggestions that HIV-1 integrase stability may be controlled by the N-end rule pathway during viral life cycle. To better assess the possible involvement of the N-end rule pathway in controlling integrase stability during retroviral infection, we generated N-terminal integrase point mutants of HIV-1 and MLV with an N-terminal stabilizing or destabilizing residue within the context of the GagPol protein (see Tables 2 and 3). For both viruses we chose methionine as the stabilizing residue since HIV-1 mutants with this residue at the N-terminus have previously been shown to produce virions (206). Both HIV-1 and MLV mature integrases bear a type 2 destabilizing residue at their N-terminus (211). Hence, we also substituted the WT destabilizing residues of both viruses with another, structurally similar type 2 destabilizing residue as an additional control. For HIV-1 we replaced phenylalanine at the N-

terminus of integrase with another aromatic amino acid, tryptophan (Table 2-2). For the integrase of MLV, we replaced isoleucine with another branched chain amino acid, leucine (Table 2-3). In order to evaluate correct packaging and maturation of viral proteins in our integrase mutants, we analyzed viral pellets by immunoblotting. As shown in Figure 2-6A, HIV-1 integrase mutants successfully produced viral particles as evidenced by the presence of the mature capsid protein (p24) in the concentrated viral pellets. However, the amount of integrase was decreased in these mutants compared to WT virus. Levels of reverse transcriptase subunits found in the viral particles paralleled the decreased levels of integrase. In contrast levels of integrase were not significantly altered for the MLV integrase mutants (Figure 2-6B).

#### **HIV-1 Integrase mutants package equivalent amounts of GagPol.**

The observed reduction in the amount of integrase and reverse transcriptase suggested a possible defect in the processing of viral GagPol polyprotein. To better assess the effect of N-terminal residue integrase mutants of HIV-1 on viral proteolytic processing, we analyzed the intravirion accumulation of Gag, GagPol and other proteolytic processing intermediates. We generated WT and integrase mutant virions in the presence of increasing amounts of an HIV-1 protease inhibitor, ritonavir. On probing with a p24 antibody, WT and mutant virions showed similar patterns of processing intermediates indicated by the decrease in

the level of p24 and p32 followed by an increase in the level of higher molecular weight products such as p55 Gag and p160 GagPol as the concentration of ritonavir increased (Figure 2-7). Gag processing is unaffected by the integrase N-terminal mutations as both WT and mutant virions showed identical patterns of cleavage intermediates (Figure 2-7, bottom). Virions produced at the highest concentrations of ritonavir (10 $\mu$ M) contained similar amounts of Gag and GagPol suggesting similar levels of packaging of these polyproteins into virions. Probing with an integrase antibody revealed similar processing intermediates p121 or p114 between wild-type and mutant virions (Figure 2-7, top). The larger proteolytic processing intermediates observed are consistent with previous studies and predictions based on the cleavage sites in the Gag and GagPol proteins (212, 213). We have observed an intermediate band between 40 and 50 kDa that appears in the presence of suboptimal ritonavir concentrations. Although this fragment may indicate an alternative cleavage site induced by the mutation at the N terminal residue of Integrase, we have not observed the same fragment in separate virus batches produced in the absence of ritonavir (data not shown). Indeed separate experiments with ritonavir have revealed a similar fragment in suboptimal concentrations of ritonavir in both mutants and the WT virus (data not shown). Additionally we observe only slight differences in both infectivity and the amount of reverse transcriptase products in different batches of virus preparations (Figure 2-8A and C). Hence this fragment is a bona fide cleavage intermediate that is not induced by the mutations at the N-terminus of

integrase. Collectively these results indicate that protease cleavage site preference is not affected by the mutations at the integrase N-terminal residue. However, in the presence of a fully active protease, the absolute levels of integrase decreased (compare total signal between WT and mutant virions at 0  $\mu$ M ritonavir) suggesting that the loss of integrase in the mutants requires HIV-1 protease activity.

### **The N-terminal mutants of HIV-1 but not MLV impact infection.**

Next, we tested the infectivity of these mutants in human cells. As shown in Figure 2-8A, infectivity was 10-20 fold lower for both HIV-1 integrase mutants compared to wild-type virus. In contrast, MLV integrase mutants exhibited slight decreases in infectivity compared to WT virus in Jurkat cells (Figure 2-8B). Notably the levels of integrase in the met or trp mutants vary (Figures 2-6, 2-7 and data not shown) but the levels of infection are equally refractory indicating that the defect is due to a function of the N-terminal amino acid in the RT/uncoating process rather than a defect in proteolytic processing.

Although the main catalytic function of retroviral integrase is to integrate viral double stranded DNA into the host cell chromosome, numerous studies have demonstrated the impact of mutations in the integrase protein on various viral

processes including uncoating (214), nuclear import (215, 216) and reverse transcription (215, 217-221). In order to determine which step(s) of the viral life cycle is affected by the HIV-1 integrase N-terminal mutants, we measured relative viral DNA accumulation at different time points following infection of the Jurkat cells using quantitative PCR analysis. As shown in Figure 2-8C, both methionine and tryptophan mutants of HIV-1 integrase showed substantially lower first jump products compared to WT integrase. Approximately 10-20 fold decrease was observed at 6 and 18 hours post infection. These results indicate that the severe inhibition of HIV-1 infection caused by the substitution of the N-terminal residue of viral integrase is the result of a block at the reverse transcription or uncoating step of the viral life cycle.

### **N-end Rule Pathway doesn't target integrase during retroviral life cycle.**

The finding that both stabilizing and destabilizing N-terminal mutants of HIV-1 exhibited similarly decreased infectivity levels supports our hypothesis that integrase is not affected by the N-end rule pathway during viral life cycle. We further tested this hypothesis in experiments using viral mutants and cellular mutants in the N-end rule. If integrase is affected by the N-end rule pathway, the stabilizing N-terminal mutant (methionine) would show similar infectivity on different N-recognition deficient MEFs, while our destabilizing mutant (Tryptophan) would behave like the WT virus. In contrast to these expectations, our mutants

showed the same pattern in their infectivity of N-recogin deficient MEFs (Figure 2-9). All HIV-1 mutants showed a 5-6 fold decrease in infectivity when all three ubiquitin ligases are depleted compared to UBR1-/- UBR2-/- cells. In contrast WT MLV and MLV integrase mutants were only slightly decreased and this could be attributed to the overexpression of shRNA since the control luciferase knockdown also resulted in a decrease in infection (Figure 2-10).

## **Discussion**

In this study we set out to investigate the role of the N-end rule ubiquitin protease degradation pathway in the retroviral life cycle. This pathway has the potential to act on viral proteins that are generated from polyproteins by protease cleavage. The resultant N-terminal amino acid can vary from the canonical methionine of most proteins and the mature protein can be subject to rapid degradation according to the N-end rule (197). In looking at HIV-1 by sequence comparison, we identified 7 mature proteins that are predicted to have a short half-life according to the N-end rule (Table 2-1). Comparing 1850 sequences at the Los Alamos HIV Sequence Database that includes all subtypes of HIV-1 and SIV<sub>cpz</sub> showed an absolute conservation for the N-terminal residue of the mature proteolytically cleaved form of 4 proteins (p1, TFP, RNaseH and Integrase). Figure 2-1 illustrates this for the integrase sequence. Since N terminal amino acid residue for these 4 proteins is under selective pressure to be conserved, we reasoned the residue at this site might interact with cellular pathways / proteins.

We initially used cell lines that were genetically inactivated for the E3 ligases involved in the N-end rule recognition N-recognins UBR1, UBR2 and UBR4 (203). From the reported overlap and redundancy in function we utilized cell lines that were multiply inactivated for combinations of the N-recognins. We show that while HIV-1 infection is impacted when all three recognins are inactivated, MLV infection is only marginally affected. This result implied that an N-end rule target protein in the host cell is involved in HIV-1 infection and its stabilization results in a reduction of infection. We also conclude that this protein interacts with HIV-1 but not with MLV and the interaction is not conserved between retroviruses. An alternative hypothesis was that the N-end rule pathway interacts with a protein or proteins from HIV-1 but not MLV. Regardless of the mode of action, we determined that the block to infection is at reverse transcription or uncoating.

In order to determine the potential viral target of the N-end rule pathway we decided to examine the role of HIV-1 proteins. We initially focused this study on the integrase molecule because it has previously been shown to be a target for the N-end rule (203, 205, 206) and it has been a focus for the development of small molecule inhibitors in recent years. Previous data had demonstrated that ectopically expressed integrase with a destabilizing residue was unstable and this instability can be rescued in N-recognin mutants (203). These studies were

extended by using ubiquitin integrase fusions that replaced the N – terminus of integrase with various amino acids and these experiments validated the action of the N-end rule again on ectopically expressed forms of integrase (203). However these studies did not determine if the integrase was a target for the N-end rule during infection since the question remained whether the N-terminus of integrase was open to interaction with N-recognins in the context of the reverse transcription complex or the pre-integration complex. These are large molecular assemblies in which integrase plays a role. One report did study the N-terminal phenylalanine of integrase in the context of viral infection by mutating the phenylalanine to the stabilizing methionine residue (206). In this study the authors also mutated the C- terminus of reverse transcriptase to compensate for any defect in proteolytic processing due to the N-terminal substitution. These authors reported that infection was greatly reduced in the methionine (and compensation) mutant and they speculate that this may be due to the stability of integrase by extrapolating the data from the previous reports (203, 205). In this study, we included an additional control by mutating the destabilizing WT residue, phenylalanine to another destabilizing and structurally conservative aromatic amino acid tryptophan. We found no absolute defect in proteolytic processing by the single amino acid mutation as reported previously (206). We found that infection was decreased for both mutations compared to the WT virus (Figure 2-8A). This result suggests that the severe inhibition of infectivity caused by the amino acid substitution at the N-terminal residue of HIV-1 integrase is not



related to the N-end rule pathway. It further confirms that the identity of the N-terminal amino acid of integrase for HIV-1 is vital to its function and explains the tremendous selection pressure that results in this phenylalanine residue being absolutely conserved. This function is unique for HIV-1 since mutating the N-terminal residues of MLV had little impact on infection of MLV.

We further observed that mutating the N-terminal amino acid in integrase resulted in intravirion instability of the integrase molecule (Figure 2-6). Intravirion instability of the members of the Pol polyprotein has been observed in previous studies (222, 223). We confirm that this instability is in part due to the action of HIV-1 protease since the stability of mutant proteins is restored by inhibiting the protease (Figure 2-7). This result suggests that HIV-1 protease may degrade the mutant proteins in the virion or that it initiates a cleavage that is acted upon by other proteases packaged into virion particles.

Since the N-terminal mutant integrase in the virion still enabled low levels of infectivity, we tested the mutant HIV-1 virions in N-recognin deficient cells. We found that all mutants had a decreased titer on the triply deficient cells compared to the UBR1 <sup>-/-</sup> UBR2 <sup>-/-</sup> cells (Figure 2-9). This reduction was not observed for MLV and mutants at the N-terminus of its integrase (Figure 2-10). These results

support our conclusion that HIV-1 integrase in the context of infection is not a target for the N-end rule pathway.

Our results also point to the importance of the first amino acid phenylalanine of HIV-1 integrase in its function. This residue is often changed using recombinant DNA techniques used to manipulate the integrase coding sequence. Typically this involves adding a methionine start codon to the protein to enable expression of the integrase molecule (224). Moreover in structural studies residual epitope tags are left as N-terminal extensions after protein purification (225). Hence future studies should aim to retain the N-terminal phenylalanine especially if integrase is studied in a complex with other proteins. Since *in vitro* assays for integrase function are efficient with recombinant N-terminal methionine bearing molecules, and we have eliminated interaction of the N-terminus with the N-end rule machinery, we speculate that an N-terminal interaction with cellular or viral components involving the phenylalanine is crucial for the early pre-integration phase of HIV-1 infection. Considering the absolute conservation of this residue, this interaction may be a potent target for small molecules to disrupt the viral life cycle.

## **Conclusions**

We conclude that HIV-1 integrase is not a direct target for the N-end rule pathway mediated degradation during infection. Further, the nature of the N-terminal residue of integrase is vital for the infectivity of HIV-1, but not MLV.

## **Materials and Methods**

### **Reagents, Plasmids and Mutagenesis**

WT, UBR4RNAi, UBR1<sup>-/-</sup>, UBR2<sup>-/-</sup> and UBR1<sup>-/-</sup> UBR2<sup>-/-</sup>, UBR1<sup>-/-</sup> UBR2<sup>-/-</sup> UBR4RNAi and UBR1<sup>-/-</sup> UBR2<sup>-/-</sup>LuciRNAi cell lines were previously established (203, 226, 227). 293T and Jurkat cells were obtained from American Type Culture Collection (ATCC). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; p24 Monoclonal Antibody (183-H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly, HIV-1 HXB2 Integrase Antiserum (aa 23-34) from Dr. Duane P. Grandgenett, HIV-1 RT Monoclonal Antibody (MAb21) from Dr. Stephen Hughes, Ritonavir. The anti-p30 to MLV was collected from a hybridoma cell line obtained from the ATCC (CRL-1912). Secondary p24 antibody for ELISA was collected from a hybridoma cell line obtained from ATCC (HB-9725). Antibody isolation from the hybridoma cell lines were performed using standard protocols (228). MLV integrase rabbit polyclonal antibody was a kind gift from Dr. Monica Roth.

Goat-anti-mouse-horseradish peroxidase and goat-anti-rabbit-horseradish peroxidase (HRP) secondary antibodies and West Femto enhanced chemiluminescent (ECL) HRP substrate were obtained from Thermo Scientific (Rockford, IL). Secondary antibody for ELISA; goat-anti-mouse-HRP IgG2A was obtained from Southern Biotech (Birmingham, AL)

Following plasmids were used in this study: CSII-EGFP; an HIV-1 based vector encoding for GFP driven by EF-1a promoter (229). CSII-DSRed; an HIV-1 based vector encoding for Ds-Red. CSII-CMVGFP; an HIV-1 based vector encoding for GFP driven by CMV promoter.  $\Delta$ NRF (230); encodes for gag, pol, rev, tat and vpu of HIV-1. pMDG (231); encodes for vesicular stomatitis virus glycoprotein. pCLMFG-GFP (232); and MLV based vector encoding for GFP, pCMVgp (232); encodes for gag and pol of MLV.

Mutations at the N terminal of HIV-1 and MLV integrase were introduced to  $\Delta$ NRF and pCMVgp respectively using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

## **Virus Production, Infections and Culture Conditions**

HIV-1 and MLV vectors were generated by transient transfection of three plasmids into 293T cells as described previously (231, 232). For HIV-1 vectors 15µg of CSII EGFP, 10µg of  $\Delta$ NRF and 5µg of pMDG were transfected using the method of Chen and Okoyama (233). 72 hours after transfection virus was collected and filtered through a 0.45 µM membrane. Following normalization via p24 ELISA, filtered virus was concentrated by ultracentrifugation (100,000 x g, 2 hours at 4°C). Viral pellet was resuspended in Phosphate buffer saline (PBS) and aliquots were stored at -80°C. Viral titers were determined by infecting  $1 \times 10^5$  Jurkat cells with 10 fold dilutions of the viral preparation. 72 hours after the infection EGFP expression was quantified by flow cytometry on a Becton-Dickinson FACScalibur. Same procedure was followed for production of MLV vectors using following plasmids; 15µg of pCLMFG-GFP, 10µg of pCMVgp and 5µg of pMDG.

MEFs and 293T cells were maintained in Dulbecco's Modified Eagle Medium (Cellgro) supplemented with 10% Fetal Bovine Serum, FBS (Gemini Bioproducts). Jurkat cells were maintained in Iscove's Modified Dulbecco's Medium (ATCC) supplemented with 20% FBS.

## Reverse transcription products qPCR assay

$1 \times 10^5$  cells were plated into 6 well dishes and infected at an MOI of 1. To control for DNA contamination, the virus was treated with 50 units/mL Benzonase (Sigma) for 1 hour at ~~37~~ before it was added to the cells. Infections were synchronized by incubating the cells 30 minutes at ~~4~~ before and after the addition of the virus. Controls consisted of uninfected cells or cells infected with heat inactivated virus for 36 hours. Cells were harvested and washed with PBS at different time points. Cell lysates were prepared by resuspending the cell pellet in lysis buffer (Tris pH 8.0, 25 mM EDTA pH 8.0, 100 mM NaCl, 1% Triton X-100, and 2 mg/ml proteinase K) and incubating at 55°C overnight. Samples were then incubated at 95°C for 15 minutes to inactivate proteinase K. Lysates were used directly for qPCR analysis. Following primers were used for qPCR(234): U31 and U32 for first jump products, MHC535 and MHC536 for 2LTR circles: 5'  $\beta$ -actin-ATC ATG TTT GAG ACC TTC AA, 3'  $\beta$ -actin-AGA TGG GCA CAG TGT GGG T, U31 - GGA TCT ACC ACA CAC AAG GC, U32 – GGG TGT AAC AAG CTG GTG TTC, MH535 – AAC TAG GGA ACC CAC TGC TTA AG, MH536 – TCC ACA GAT CAA GGA TAT CTT GTC. QPCR reactions using SYBR green were performed using Eppendorf real plex master cycler ep and BioRad SYBR SuperMix following the manufacturers protocol. Cycling conditions used were 95°C for 2 min, followed by 40 cycles of 95°C 30s, 58°C 30s, and 72°C 30s, and a final extension of 5 minutes at 72°C for all PCR products. Cycle threshold value was used to normalize the DNA amounts to the first time point of the control

sample. The melt curve as well as analysis of the PCR products by agarose gel electrophoresis confirmed the presence of one product at the expected size (data not shown). DNA input was controlled by qPCR amplification of a fragment of the  $\beta$ -actin gene.

### **Enzyme-linked immunosorbent assay (ELISA)**

A 96 well plate (NuncMaxisorp, Fisher Scientific) was coated with a primary p24 antibody (183-H12-5C) in coating buffer (100 mM Sodium Bicarbonate, pH 8.5) and incubated overnight. Wells were then washed 3 times with Phosphate Buffer Saline-Tween (PBS-T) and blocked for 1 hour using 5% milk in PBS-T at room temperature. Following a 3x wash with PBS-T, viral samples were added to the wells at different concentrations in Sodium Chloride-Tris-EDTA (STE) buffer-empigen (0.1 M NaCl, 10mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1% empigen) in triplicates and incubated at 37°C for 1 hour. Wells were then washed 3x with PBS-T and secondary p24 antibody (31-90-25) was added to each well and incubated at room temperature for 1 hour. Following a 3x wash with PBS-T goat-anti-mouse IgG2A-HRP was added to the wells and incubated for 30 minutes at room temperature. Wells were then washed 3x with PBS-T and 3,3',5,5'-Tetramethylbenzidine, TMB (Sigma) was added to the wells. Following a 10 minute incubation at room temperature, the reaction was stopped with 2N

H<sub>2</sub>SO<sub>4</sub>. Absorbance was determined at 450 nm using a microplate reader (BioTEK, SynergyMX).

### **Immunoblotting for viral proteins**

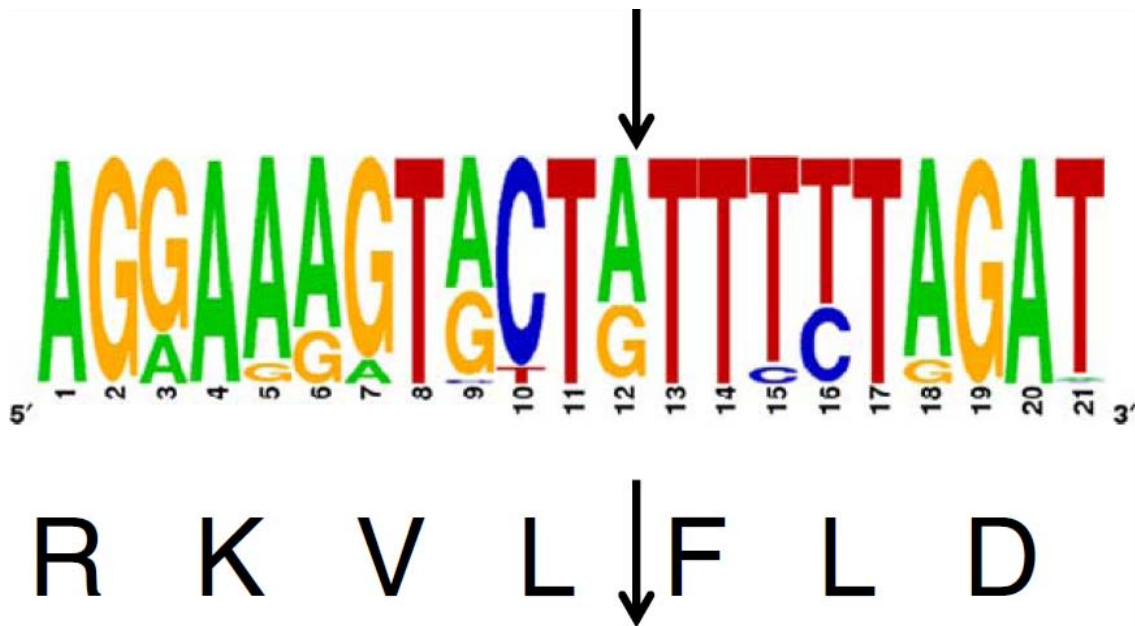
Filtered virus containing supernatants were concentrated as described above. Following normalization for p24 via ELISA, viral pellets were solubilized in loading buffer (0.25M Tris-HCl, pH 6.8, 15% SDS, 50% glycerol, 25%  $\beta$ -mercaptoethanol, 0.01% bromophenol blue) and proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and subjected to immunoblotting using the antibodies indicated.

### **Analysis of intravirion proteolytic processing of HIV-1 polyproteins**

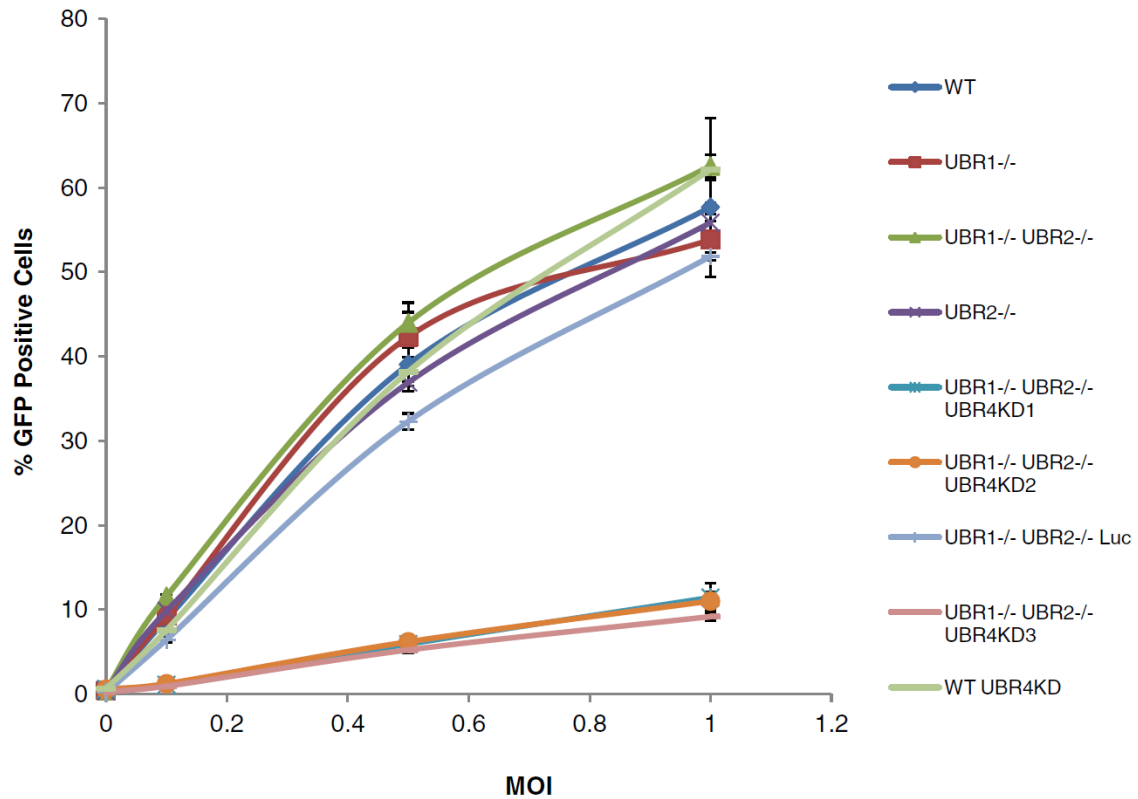
293T cells ( $1 \times 10^6$ ) were transfected using the method of Chen and Okoyama(233) with 15 $\mu$ g of CSII EGFP, 5 $\mu$ g of pMDG, and 10 $\mu$ g of  $\Delta$ NRF which contains different mutations corresponding to the relevant integrase N-terminal mutant. 8 hours later transfection medium was replaced with culture medium containing different concentrations of the HIV-1 protease inhibitor, ritonavir. 48 hours later virus was collected, filtered and centrifuged at 100,000xg for 2 hours



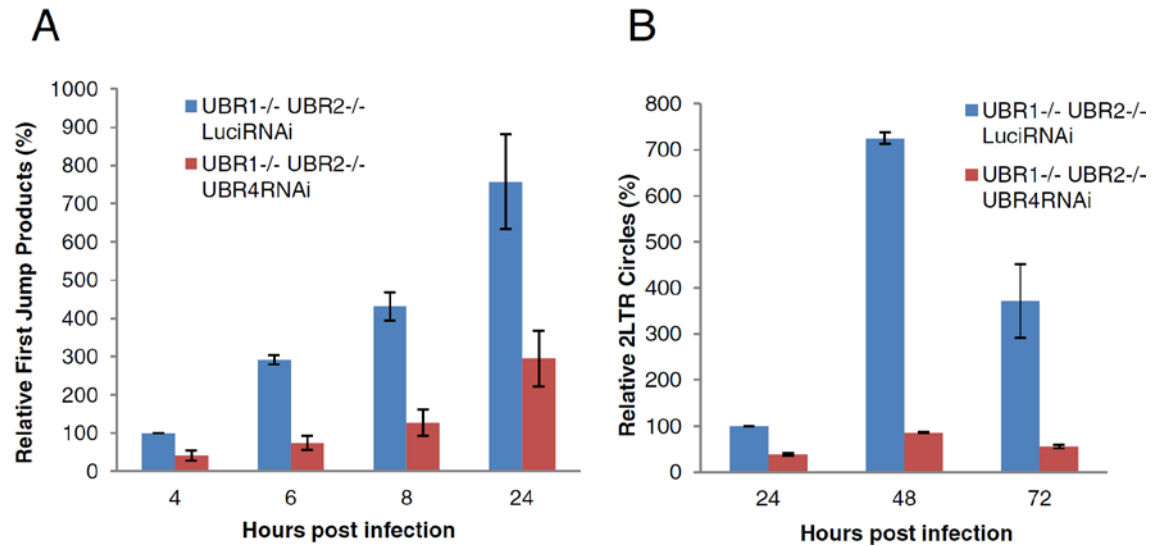
at 4°C. Purified virions were quantified by p24 ELISA and protein composition of the virions was assessed by immunoblotting as described above.



**Figure 2-1. N terminal residue of HIV integrase is highly conserved.** Analysis of sequence of the protease cleavage site between RT and integrase of 1850 isolates of HIV-1 and SIVcpz present in Los Alamos HIV Database (<http://www.hiv.lanl.gov>) using the web alignment tool. Corresponding amino acid for the conserved sequence is shown at the bottom. The sequence logo at the top was generated using WebLogo (<http://weblogo.berkeley.edu/>). Arrows indicate the protease cleavage site.



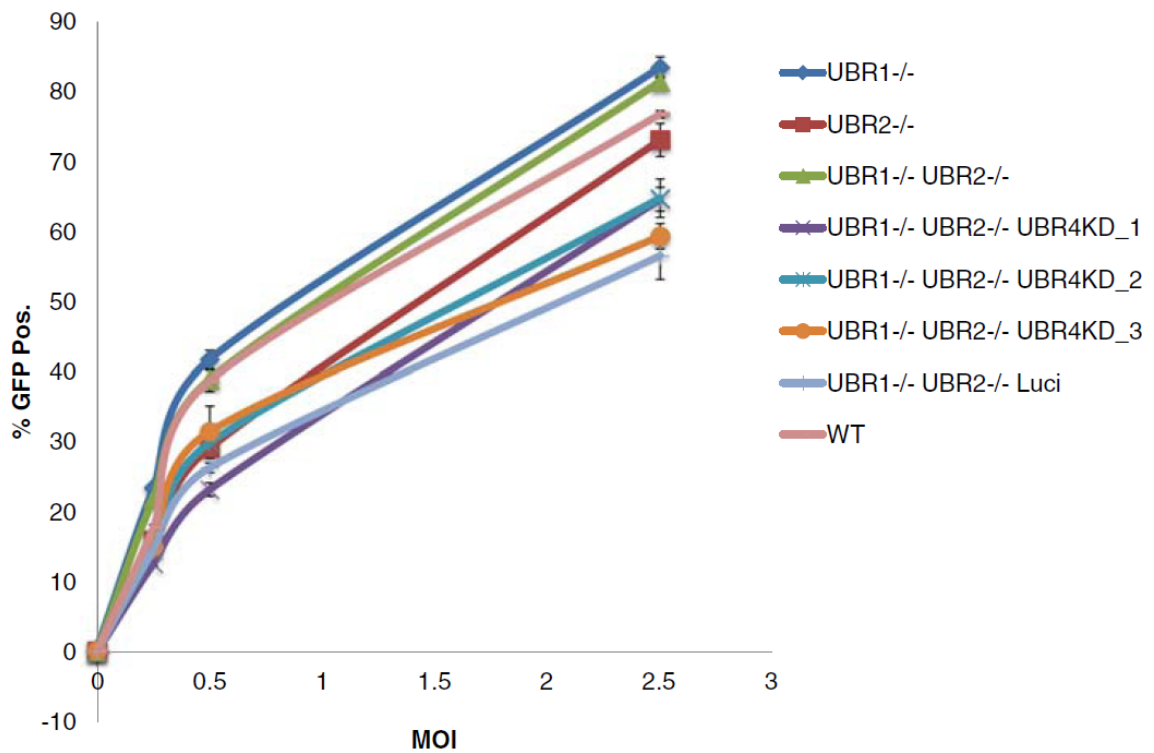
**Figure 2-2. HIV-1 infectivity is decreased when all three N-recognins are depleted.** WT and N-recognin deficient MEFs were infected with VSVG pseudotyped HIV-1-EGFP at an increasing MOI of 0.1, 0.5 and 1. The percent of GFP positive cells were determined by fluorescence cytometry 3 days post infection.



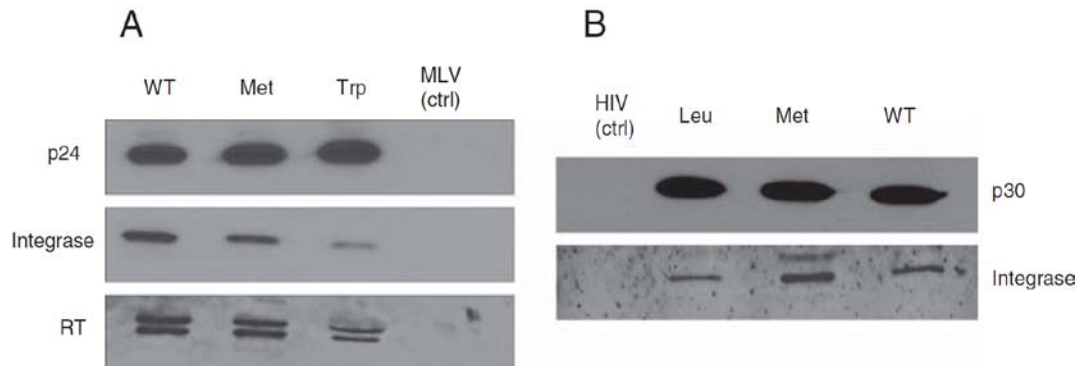
**Figure 2-3. Kinetics and nuclear localization of proviral DNA in N-recognin deficient cells.** UBR1-/- UBR2-/-LuciRNAi(control) and UBR1-/- UBR2-/-UBR4RNAi cells were infected with VSVG pseudotyped HIV-1-EGFP at an MOI of 1. Viral cDNA products were quantified by qPCR at indicated times. (A) First jump products. The data are expressed as relative to the control 4hr time point. (B) 2LTR circles. The data are expressed as relative to the control 24hr time point. The data for the UBR1-/- UBR2-/-UBR4RNAi are the average of UBR1-/- UBR2-/-UBR4KD1 and UBR1-/- UBR2-/-UBR4KD2 cells.

Virus	Amino acid at integrase N-terminus	N-end rule designation
<b>Lentiviruses</b>		
HIV-1	Phe	Primary Destabilizing
HIV-2	Phe	Primary Destabilizing
SIV	Phe	Primary Destabilizing
EIAV	Trp	Primary Destabilizing
<b>Alpharetroviruses</b>		
MAV	Pro	Stabilizing
RSV	Pro	Stabilizing
<b>Betaretroviruses</b>		
MMTV	Ser	Stabilizing
MPMV	Ser	Stabilizing
<b>Gammaretroviruses</b>		
MLV	Iso	Primary Destabilizing
<b>Deltaretroviruses</b>		
HTLV	Ser	Stabilizing
<b>Epsilonretroviruses</b>		
WDSV	Val	Stabilizing
<b>Spumaretroviruses</b>		
HFV	Cys	Secondary Destabilizing

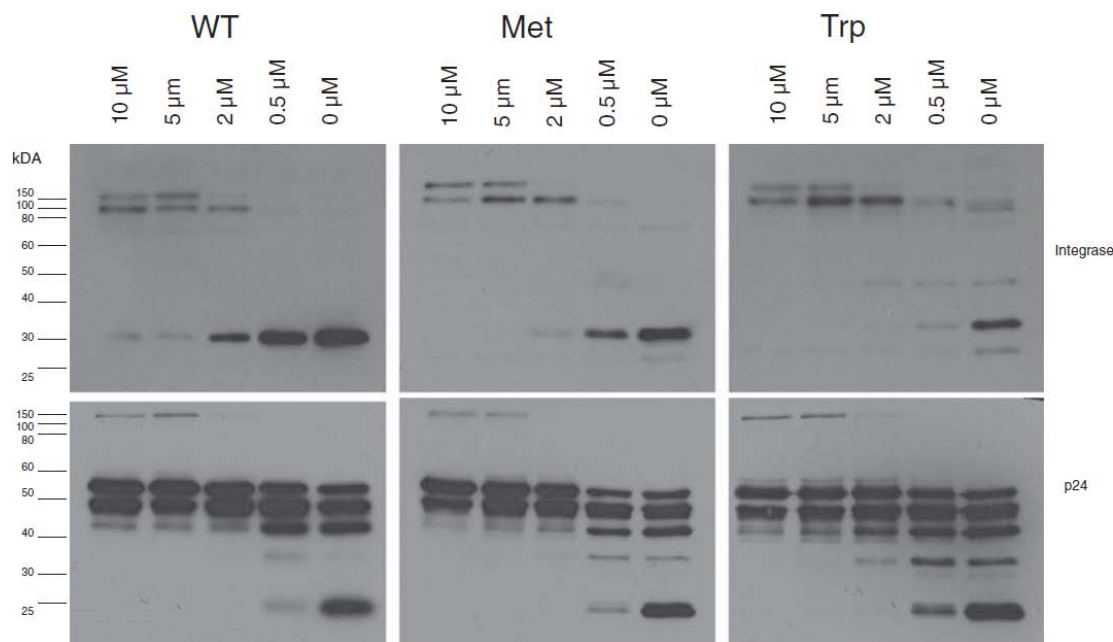
**Figure 2-4. Only some retroviral integrases bear a N-terminal type-2 destabilizing residue.** A comparison of the identity and nature of the amino acid at the N-terminus of mature integrase for various retroviruses. Representatives of the family of retroviruses were chosen based on sequence characterization of the cleavage site or the integrase protein from the literature (207, 211, 212, 235-242)



**Figure 2-5. MLV infectivity is not affected in N-recognin deficient cell.** WT and N-recognin deficient MEFs were infected with VSVG pseudotyped MLV-EGFP at an increasing MOI of 0.25, 0.5 and 2.5. The percent of GFP positive cells were determined by fluorescence cytometry 3 days post infection.

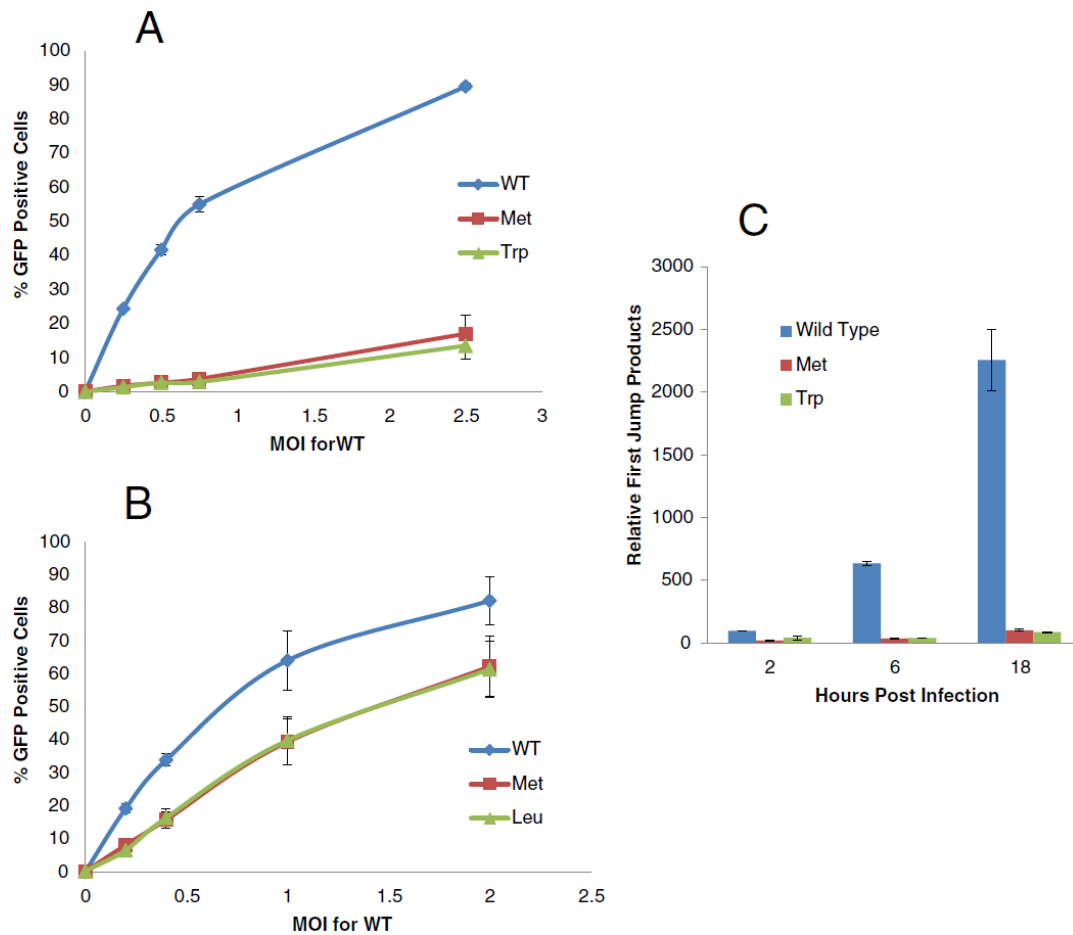


**Figure 2-6. Intravirion integrase levels are decreased in HIV-1 integrase N-terminal mutants.** HIV-1 or MLV virions were produced in 293T cells using helper plasmids with either WT or engineered mutations for the N-terminus of mature integrase. Virions particles were concentrated and capsid equivalent (p24 for HIV-1 and p30 for MLV) extracts were analyzed by immunoblot analysis. **A)** HIV-1 vector particle extracts probed with p24, integrase or RT antibodies. The MLV (ctrl) particle extract serves as a negative control. **B)** MLV vector particle extracts probed with p30 or MLV integrase antibodies. The HIV (ctrl) extract serves as a negative control.

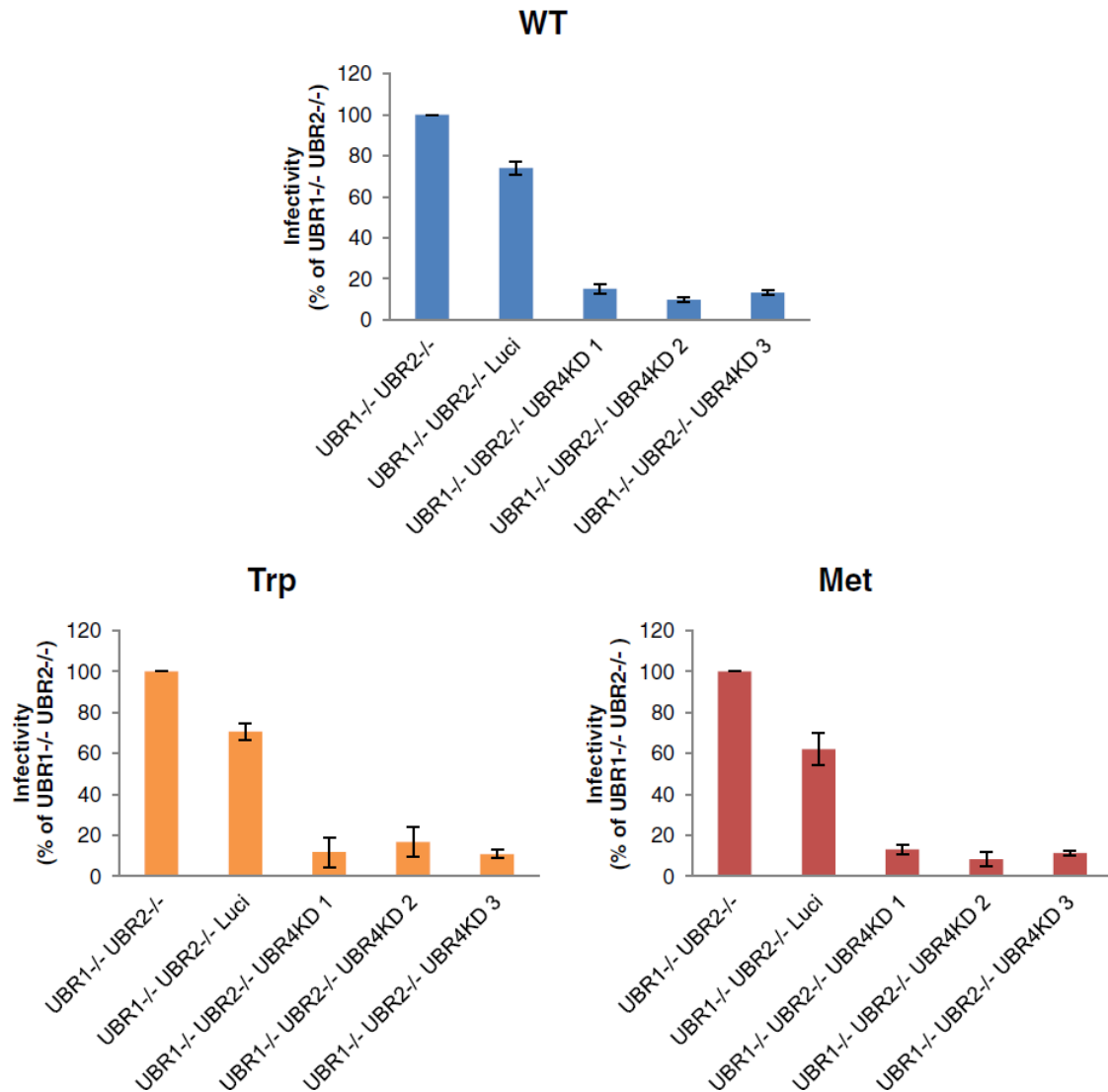


**Figure 2-7. Intravirion processing of Gag and Gag-Pol polyproteins in HIV-1 integrase N-terminal mutants.** WT and mutant HIV-1 virion particles were produced in the presence of varying concentrations of the HIV-1 protease inhibitor ritonavir and the cleavage pattern of the Gag and GagPol polypeptides were analyzed by immunoblot analysis. WT, methionine substituted (Met) and tryptophan substituted (Trp) integrase mutants were probed with antibodies to integrase (top) or p24 (bottom).

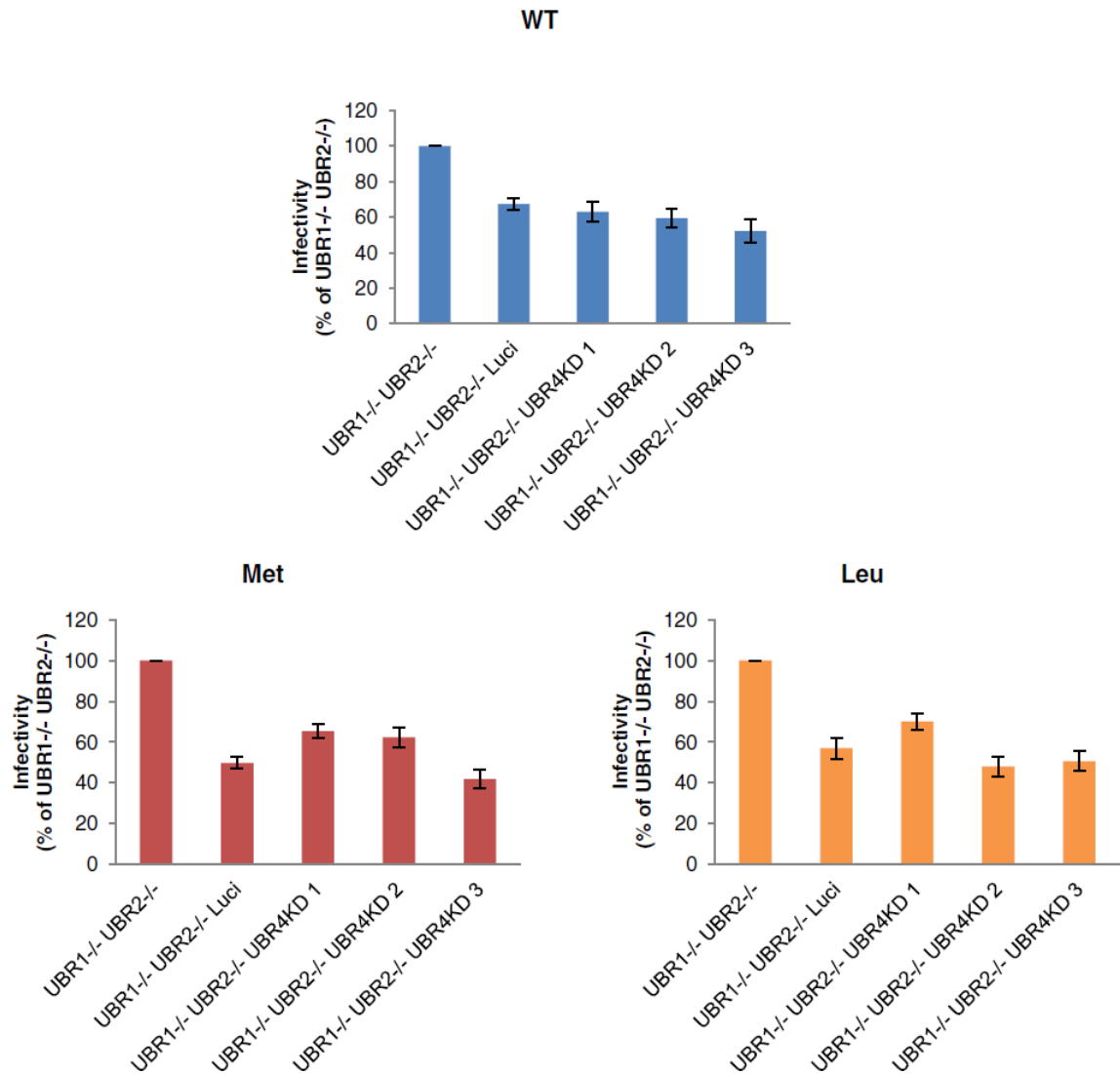




**Figure 2-8. Effect of N-terminal Integrase mutations on HIV-1 and MLV viral infectivity.** WT or mutant integrase HIV-1 (A) or MLV (B) viral vectors equilibrated to p24 (HIV-1) or p30 (MLV) amounts were incubated with Jurkat cells and infectivity was measured by flow cytometry 3 days after infection. MOI for WT was measured as indicated in the Methods section and the viral amounts corresponding to equivalent p24 or p30 levels were used for integrase mutants (C) HIV-1 vectors at an MOI of 1 were incubated with Jurkat cells and viral cDNA first jump products were quantified by qPCR at indicated times. The data are expressed as relative to the control (wild-type) 2hr time point.



**Figure 2-9. HIV-1 Integrase N-terminal mutants behave similarly in N-recognin deficient cells.** WT and integrase mutant (Trp and Met) HIV-1 vectors were used to infect N-recognin deficient cells. The infectivity was measured by flow cytometry 3 days post infection and is expressed relative to infection of the UBR1-/- UBR2-/- cell line.



**Figure 2-10. MLV Integrase N-terminal mutants behave similarly in N-recognin deficient cells.** WT and integrase mutant (Leu and Met) MLV vectors were used to infect N-recognin deficient cells. The infectivity was measured by flow cytometry 3 days post infection and is expressed relative to infection of the UBR1-/- UBR2-/- cell line.

HIV-1 Cleavage Site and conservation	Amino Acid at the N terminus	N-end Rule Designation
MA/CA Conserved	Proline	Stabilizing
CA/p2 Conserved	Alanine	Stabilizing
P2/NC Variable	Methionine	Stabilizing
<b>NC/p1 Conserved</b>	<b>Phenylalanine</b>	<b>Primary Destabilizing</b>
p1/p6(gag) Variable	Leucine	Primary Destabilizing
<b>NC/TFP Conserved</b>	<b>Phenylalanine</b>	<b>Primary Destabilizing</b>
TFP/p6(pol) Variable	Leucine	Primary Destabilizing
p6(pol)/PR Variable	Proline	Stabilizing
PR/RT Conserved	Proline	Stabilizing
<b>RT/RH(p15) Conserved</b>	<b>Tyrosine</b>	<b>Primary Destabilizing</b>
<b>RH(p15)/INT Conserved</b>	<b>Phenylalanine</b>	<b>Primary Destabilizing</b>
Nef Variable	Leucine	Primary Destabilizing

**Table 2-1. HIV-1 proteins have N-terminal destabilizing residues**

Comparison of 84 protease cleavage site sequences (27 C-type, 30 B-type and 27 M-type)(208). The position and the conservation relatives to the most common recent common ancestor (MRCA) are shown. The proteins that are conserved for a destabilizing residue as confirmed by an analysis of 1850 isolates of HIV-1 and SIVcpz present in Los Alamos HIV Database (<http://www.hiv.lanl.gov>) are highlighted in bold.

Cleavage site (RH/INT)	N-end Rule Designation	Amino acid at P1'
RKVL/ <u>E</u> LD (WT)	Destabilizing	Phenylalanine
RKVL/ <u>M</u> LD	Stabilizing	Methionine
RKVL/ <u>W</u> LD	Destabilizing	Tryptophan

**Table 2-2. N-terminal integrase mutants of HIV-1.**

Cleavage site (RH/INT)	N-end Rule Designation	Amino acid at P1'
STLL/ <u>I</u> EN (WT)	Destabilizing	Isoleucine
STLL/ <u>M</u> EN	Stabilizing	Methionine
STLL/ <u>L</u> EN	Destabilizing	Leucine

**Table 2-3. N-terminal integrase mutants of MLV.**

## **Chapter 3**

**Nature of the N-terminal Amino Acid  
Residue of HIV-1 RNase H is Critical  
for the Stability of Reverse  
Transcriptase in Viral Particles**

## Summary

Reverse transcriptase (RT) of Human Immunodeficiency Virus type 1 (HIV-1) is synthesized and packaged into the virion as a part of the GagPol polyprotein. Mature RT is released by the action of viral protease. However unlike other viral proteins RT is subject to an internal cleavage event leading to the formation of two subunits in the virion – a p66 subunit and a p51 subunit that lacks the RNase H domain. We have previously identified RNase H as an HIV-1 protein that has the potential to be a substrate for the N-end rule pathway which is a ubiquitin dependent proteolytic system in which the identity of the N-terminal amino acid determines the half life of a protein. Here we examined the importance of the N-terminal amino acid residue of RNase H in the early life cycle of HIV-1. We show that changing this residue to a structurally different amino acid than the conserved residue leads to degradation of RT and in some cases integrase in the virus particle and this abolishes infectivity. Using intravirion complementation assays with Vpr fusion proteins we show that liberation of RNase H mutant RT from GagPol is necessary for this degradation. Our data also suggests that association of RT subunits is impaired in at least one of the RNase H N-terminal mutants. These results indicate the importance of the N-terminal amino acid residue of RNase H in HIV-1 viral fitness.



## **Introduction**

Like all retroviruses Human Immunodeficiency Virus Type (HIV-1), the causative agent of AIDS, synthesizes and packages its main structural and enzymatic proteins as precursor polyproteins. For HIV-1 these polyproteins are p55 (Gag) and p160 (GagPol). Gag is the most abundant polyprotein and is translated from a genomic length mRNA that contains the Gag and GagPol open reading frames. The synthesis of GagPol requires a ribosomal frameshift leading to a Gag:GagPol ratio of about 20:1 in the virus particle (18). Individual mature viral proteins are generated following viral assembly as a result of a series of proteolytic cleavage events at specific positions catalyzed by the viral protease which is synthesized as a part of GagPol (48).

One protein that is released as a result of proteolytic processing of GagPol is reverse transcriptase (RT). RT catalyzes the reaction for the conversion of viral RNA to double stranded DNA (243). In contrast to the other viral enzymes encoded by the pol gene, RT functions as a heterodimer of two subunits, p66 and p51 (244-246). Formation of this heterodimer requires the proteolytic cleavage of the RNase H domain from one of the p66 subunits resulting in p51 that is associated with the p66 to form the heterodimer (244). RNA dependent DNA polymerase, and RNase H activities of HIV-1 RT are mainly carried out by the p66 subunit, while p51 was thought to be enzymatically inactive and only serving a structural role (245, 247-250). However, recent structural and

biochemical evidence suggests that C-terminal end of the p51 subunit is involved in hydrolysis and positioning of the RNA/DNA hybrid formed during the reverse transcription process (251-253).

Retroviral RNase H is a member of a family of enzymes that are found in all domains of life (254). It functions as an endonuclease which degrades RNA from the RNA/DNA hybrid formed during the first phase of reverse transcription. This function is crucial for the processing and completion of reverse transcription, as it creates an RNA primer for plus strand DNA synthesis and as it facilitates the first and second jump by removing the 5' end of viral RNA and tRNA respectively (248, 255, 256). In the virus particle RNase H is found both as a part of p66 and as a free protein (244). However it is not definitively established whether the RNase H species that is cleaved off by the viral protease to create the RT heterodimer has any specific function.

The N-end rule pathway is an ubiquitin dependent proteolytic system in which the identity of the N-terminal amino acid determines the half life of a protein. Since proteolytic cleavage of viral polyproteins can result in N-terminal residues that determine a short half life for the cleaved protein according to this rule we have recently examined the involvement of the N-end rule pathway in the retroviral life cycle. Using N-end rule mutant cells and N-terminal amino acid substitution

mutants we studied the effects on the mature integrase protein that bears a highly conserved N-terminal destabilizing residue (257). Our results showed an impact of the N-end rule pathway on the HIV-1 but not MLV life cycle. However the interaction was not at the level of the integrase N-terminal residue (257). One of the differences in the protein composition of HIV-1 and MLV is that unlike the heterodimeric RT of HIV-1, MLV RT functions as a monomer of about 75kDa protein, hence doesn't require a proteolytic cleavage to remove the RNase H domain (258, 259). Due to this difference between HIV-1 and MLV and the fact that HIV-1 RNase H also has the potential to be a substrate for the N-end rule pathway, we turned our attention to this protein.

In this study we investigated the role of the N-terminal amino acid residue of HIV-1 RNase H. Here we show that the N-terminal residue of RNase H is highly conserved and changing this residue to an amino acid that is structurally different than the WT residue leads to degradation of RT and in some cases integrase in the virus particle. Notably this degradation in the virus particle is independent of the N-end rule that would manifest in the target cell. We demonstrate that this degradation is processive and does not extend to RT species that are added in trans into the viral particle. Our results also indicate that liberation of RT from GagPol or a Vpr fusion protein is required for the degradation of RNase H N-terminal mutants. Moreover we show that N-terminal RNase H mutant RT is not degraded *in vitro* in the presence of excess viral protease. We also present

evidence that at least one of the RNase H N-terminal mutants have a defect in association of RT subunits.

## **Results**

### **N-terminal amino acid residue of HIV-1 RNase H is highly conserved**

In our previous study we have reported on 7 HIV-1 mature proteins as having primary destabilizing residues as defined by the N-end rule (257). Further analysis indicated that only 4 of these proteins (p1, Trans frame octapeptide, RNase H and Integrase) have a conserved residue at the N-terminus. Here we investigate the role of the N-terminal residue of RNase H during HIV-1 infection. Comparison of the sequence of the cleavage site between the p51 subunit of RT and RNase H in HIV-1 isolates present in Los Alamos HIV sequence database reveals that the sequence for the N-terminal amino acid residue of RNase H is absolutely conserved for all the isolates found in this database (Figure 3-1). This suggests a hypothesis that changing this residue would have deleterious effects on viral infectivity.

### **RNase H N-terminal mutations impact intravirion protein levels and viral infectivity**

To test the role of the N-terminal residue of RNase H in the viral life cycle of HIV-1, we changed this residue to several different amino acids. Selection of mutants

was based on N-end rule designation or structure of the specific amino acid as compared to the wild type (WT) residue of Tyrosine (Table 3-1). We first analyzed the effect of these N-terminal RNase H mutations on viral protein packaging and maturation by immunoblotting. We observed that viral pellets of most of the RNase H mutants contained drastically reduced levels of RT as compared to WT viral pellets (Figure 3-2). Extended exposure of the immunoblot (using enhanced chemiluminescence for detection) revealed that some mutant (i.e. Proline (Pro) and Lysine (Lys)) virions contained barely detectable levels of RT, while others contained diminished amounts of p51 (data not shown). In contrast, two of the mutants (Tryptophan (Trp) and Phenylalanine (Phe)) containing structurally similar amino acids to the WT tyrosine residue had slightly reduced levels of RT compared to WT (Figure 3-2, top blot). Since we didn't observe any low molecular weight peptide fragments using a monoclonal RT antibody (Figure 3-2), we also tested some of our mutants with a polyclonal RT antibody to detect possible degradation products (data not shown). While this analysis revealed the appearance of a predominant immunoreactive peptide of between 40 and 30 kDa as well as some minor low molecular level bands in three of the mutants (Methionine (Met), Trp and Phe), these peptide fragments are not apparent in mutant virions that have drastically decreased levels of RT (data not shown). We didn't observe any significant difference between RNase H mutants and WT in Gag or GagPol protein production in virus producing cells that can explain the lack of intravirion RT (data not shown). We further probed our

RNase H mutants for the presence of other HIV-1 proteins, and this analysis revealed that some of the mutants also had diminished levels of intravirion integrase (Figure 3-2, middle panel). The mutants that had the most effect on the RT levels (i.e. Pro and Lys) showed the most pronounced decrease in virion integrase levels as well. We further probed the RNase H mutant virions for viral capsid protein (p24) to determine whether the mutations we introduced caused any gross aberrations in assembly or processing. As shown in the bottom panel of Figure 3-2, a product of the Gag processing, p24, is found in all mutants suggesting WT levels of protease activity on the Gag polyprotein in the virions.

Next, we tested the infectivity of the N-terminal RNase H mutants of HIV-1. As shown in Figure 3-3, infectivity was slightly decreased (by a factor of 0.75) for Trp and Phe mutants that were not compromised for RT levels in the virions. Infectivity was undetectable in all the other mutants tested except for the Met mutant which had about 100 fold decrease in infectivity compared to WT.

### **Packaging and processing of viral polyproteins with RNase H N-terminal mutations**

Despite the correct processing of Gag to p24 in all of the N-terminal RNase H mutants tested (Figure 3-2 bottom blot), diminished levels of RT and integrase in some mutants indicate the possibility of aberrant packaging or aberrant processing of GagPol. To better assess this, we analyzed the presence of

GagPol, Gag and other proteolytic processing products in the mutant virions. We produced WT and RNase H mutant virions (Met and Leu which are typical for residual RT of other mutants) in the presence of varying amounts of ritonavir, an HIV-1 protease inhibitor. Following concentration, virions were analyzed by immunoblotting. As shown in Figure 3-4 bottom panel, WT and mutant virions showed similar patterns of cleavage intermediates when probed for p24. As the concentration of ritonavir increased the levels of processed p24 decreased with a corresponding increase in the amounts of higher molecular weight products such as Gag and GagPol (Figure 3-4, bottom panel). Notably at the highest concentration of ritonavir the Gag and GagPol levels were similar in all virions suggesting that the mutants do not compromise assembly of these into virion particles. When probed with RT antibody, cleavage patterns of WT and mutant virions are only comparable at the higher ritonavir concentrations (Figure 3-4, top blots). For virions produced in the presence of 1 or 0.5  $\mu$ M of ritonavir, WT virus shows the predicted processing intermediates consistent with previous reports (212, 213). There is a loss of immunoreactive epitopes in the two mutants examined (Figure 3-4 top panel). From this analysis we cannot conclude whether a higher molecular weight intermediate or the RT p66 species is mainly susceptible to the degradation observed in the mutants. However the appearance of similar levels of GagPol and Gag in both mutants and WT at the highest ritonavir level indicates correct packaging of these polyproteins in RNase H N-terminal mutants.

## **Delivery of RT independent of GagPol into HIV-1 virions**

Our analysis of proteolytic processing in RNase H mutants did not allow us to conclude whether this degradation requires only the RT p66 species or a higher molecular processing intermediate. In addition we wondered if this degradation can affect WT RT p66 species that may dimerize with a mutant RT if both were present in the virion. To test these questions we delivered p66 synthesized independent of GagPol into virions containing WT or RNase H mutant GagPol. This was accomplished by using fusions to the HIV-1 accessory protein vpr that is packaged into the virion by binding to the p6 domain of Gag (260). We generated constructs by fusing vpr to WT or RNase H mutant RT-Integrase (Figure 3-5A and B). The protease cleavage site (PC) between protease and RT to the end of the vpr was retained to enable cleavage and release of RT-Integrase in the virus particle. We generated WT or N-terminal RNase H mutant viral particles in the presence or absence of vpr fusion constructs. Concentrated virions were analyzed by immunoblotting following p24 ELISA to enable equivalent loading based on viral capsid concentration. As shown in Figure 3-6 top panel, when probed with a vpr antibody, WT or mutant viruses complemented with vpr-RT-INwt fusion constructs contain the predicted 4 bands as a result of protease cleavage at 3 different sites; between vpr and RT, p51 and RNase H and RNase H and integrase (Figure 3-6 top panel: lanes marked +vpr-RT-INwt). When we probed vpr-RT-IN complemented mutant virions with an RT antibody we observed the almost complete rescue of both RT subunits only when the vpr



constructs contained WT or Trp mutant RNase H fusions (Figure 3-6A and C middle panel). RNase H N-terminal mutants that are structurally different than the WT showed either diminished levels of RT subunits (Figure 3-6C) or the appearance of only low levels of p51 (Figure 3-6A). Notably the vpr fusions appear to be at similar levels in virion particles (Figure 3-6 top panel and middle panel) and the RT subunits are subject to degradation suggesting that these are the substrates for proteolysis. Similar to the results obtained with the vpr-RT-Integrase fusions, we observed the appearance of both RT subunits only in the virions complemented with vpr-RTwt or vpr-RTtrp when we delivered vpr-RT fused RNase H mutants (data not shown). These results support the idea that the mutant RT p66 is the substrate for proteolysis once it is liberated from the polyprotein (GagPol or from the vpr fusion).

### **Infectivity of Vpr-RT-Integrase complemented viruses**

Since the vpr-RT-IN complemented RNase H N-terminal mutants showed rescue of intravirion RT when complemented with WT or Trp mutants, we tested if this molecular rescue also extends to infectivity of the RNase H mutants. We infected Jurkat cells with WT or mutant vpr-RT-IN complemented viruses (Figure 3-7). As predicted by immunoblotting results, infectivity of Leu, Met and Pro mutants were rescued to similar levels by complementation with vpr-RT-INwt (Figure 3-7B, C and D). We also observed rescue of infectivity by vpr-RT-INtrp, albeit at different

levels for each mutant (Figure 3-7B, C and D). In contrast, complementation with vpr-RT-IN constructs containing RNase H mutants that had diminished levels of RT in the virion did not rescue infectivity in any of our mutants (Figure 3-7B, C and D). Of note there is a general slight decrease in infectivity in WT virions that are complemented with different vpr constructs.

### **RNase H N-terminal mutant RT is stable *in vitro***

Our observations show that RT of most of the N-terminal mutants of RNase H are degraded in virions and that this degradation is dependent on release of the mutant p66 from the polypeptide in which it is embedded (Figure 3-4 for GagPol, Figure 3-6 for vpr-RT-Integrase). However, it is not clear whether this degradation is caused by the viral protease (for example by the mutation uncovering cryptic target sites) or a cellular protease that is packaged into the virion. We first investigated this by using a rabbit reticulocyte lysate (RRL) *in vitro* transcription/translation system. Since Gag is the dominant protein species translated from full length viral mRNA this would interfere with our ability to detect RT that is made from the frameshifted GagPol polyprotein (18). Hence we used a GagPol transcript with an engineered frameshift that would produce only full length GagPol polyprotein (Figure 3-5C). We further introduced an N-terminal RNase H mutation (Pro) to this construct since it is the mutant with the most

drastic effect on RT stability in the virion. Previous studies on *in vitro* translated GagPol polyprotein showed that the encoded protease cannot completely process the GagPol precursor to mature viral proteins (213, 261, 262). A strong initial cleavage event between p2 and nucleocapsid (NC) was observed followed by minor cleavages at other sites (213). However, addition of HIV-1 protease to the reaction has been shown to generate mature viral proteins (261). In our assay system we first translated the GagPol *in vitro* and then incubated the final product with the exogenous HIV-1 protease and used immunoblot analysis to probe for RT release and stability. As shown in Figure 3-8A, in the absence of exogenous protease we observe the predicted proteins that result from a single dominant cleavage of GagPol by the intrinsic protease (40 kDa, and an approximately 120 kDa). We also observed lower molecular weight peptides using this RT monoclonal antibody, consistent with several minor cleavage products observed previously (213, 262). Consistent with previous findings, addition of HIV-1 protease resulted in the processing of GagPol with the appearance of p24 for both WT and the RNase H proline mutant, when probed with a p24 antibody (Figure 3-8A, top blot). We also observed the appearance of the p51 and p66 subunits of RT from WT GagPol following incubation with the viral protease (Figure 3-8A, bottom blot). In contrast to the intravirion degradation of RT in the RNase H proline mutant, the *in vitro* maturation of GagPol containing the RNase H proline mutant resulted in stable RT subunits (p51 and p66) at similar levels to WT levels (Figure 3-8A, bottom blot). Addition of greater

concentrations of viral protease or longer incubations times did not reveal any additional cleavages of the final products (data not shown).

### **RNase H N-terminal Proline mutant causes impairment in dimerization of RT subunits**

The above in vitro experiments suggest that viral protease may not be responsible for the degradation of intravirion RT of some RNase H N-terminal mutants, there remains the possibilities that the contents of the viral milieu, concentration of viral protease or specific interactions between the viral proteins inside the virion are required for the mutant RT to be degraded in the virus particle. To properly investigate these possibilities we packaged the p66 RT subunit into virions without the need to fuse it to any protein. This would enable a test of intravirion stability of RT in the absence of an active viral protease. To this end we utilized a previously described construct pLR2P-vpr-p51-IRES-p66 (vpr-p51/p66) (263). In this construct, vpr fused p51 subunit is packaged into the virion through the interaction of vpr with the p6 domain of Gag, while the p66 subunit is packaged via its interaction with the p51 subunit in the vpr-p51 fusion (263). Since the vpr-p51/p66 construct makes it difficult to distinguish between vpr-p51 fusion protein and p66 on immunoblots probed with an RT antibody (264), we generated a construct that contains 50 amino acids from the C terminus of HIV-1 protease between the vpr and p51 (Figure 3-5D). This enabled

us to distinguish between vpr-pro50-p51 and p66 on immunoblots. To test for the involvement of viral protease in the degradation of RT in RNase H mutants, we inactivated the viral protease by introducing a D25A mutation into the protease gene in our viral production helper plasmid  $\Delta$ NRF. Using these constructs we produced WT or RNase H mutant vpr-pro50-p51-IRES-p66 complemented virions, and analyzed them by immunoblotting. As shown in Figure 3-8B, both WT and Trp mutant complemented protease inactive virions contain the expected peptides for vpr-pro50-p51 and p66 when probed with an RT antibody. In contrast, RNase H Pro mutant complemented virions lack the peptide for the p66 subunit and only contain the vpr-pro50-p51 peptide (Figure 3-8B, top blot). Because p66 is translated and packaged to the virion as a separate (non fusion) protein in the vpr-p51/p66 construct we wanted to determine whether it is stably expressed in the cells used to produce the virus. To this end we analyzed extracts of 293T cells co-transfected with WT or RNase H mutant vpr-pro50-p51-IRES-p66 constructs and  $\Delta$ NRF D25A. As shown in Figure 3-8C, both vpr-pro50-p51 fusion protein and p66 are expressed from WT and RNase H mutant (Trp and Pro) containing constructs. Of note we observe two bands related to p66 in our mutants when expressed in 293T cells (Figure 3-8C). Similar p66-related peptides were previously detected by others when mutations introduced into RT were close to the dimer interface and were thought to be generated by the action cellular proteases (265, 266). Even though these results indicate that RNase H proline mutant p66 is expressed in the virus producing cells, there still remains

the possibility that it is not packaged into the virions due to a defect in binding to vpr-pro50-p51 caused by the mutation. To test this possibility we performed a co-IP assay. For this we incubated extracts of 293T cells co-transfected with  $\Delta$ NRF D25A and vpr-pro50-p51 $\Delta$ p66, vpr-pro50-p51/p66wt or vpr-p51/p66pro with a vpr antibody. Bound protein complexes were pulled down with Protein G conjugated beads and analyzed by immunoblotting. As shown in Figure 3-8D top panel, both vpr-pro50-p51 and p66 can be detected in both the lysate (Inp) and immunoprecipitate (IP) from the cells expressing WT p66 (lanes under pLR2P WT). In contrast, p66 is only present in the lysate and not in the immunoprecipitate of the cells expressing RNase H proline mutant p66 (Figure 3-8D top panel, lanes under pLR2P Pro). These results indicate that dimerization of RT p66 with p51 is impaired when the N-terminal residue of RNase H is changed to Proline.

## **Discussion**

RNase H of HIV-1 is cleaved out of about half of the viral p66 RT species that are synthesized and this results in the association of heterodimeric subunits of RT, p51 and p66 (244). This proteolytic cleavage releases RNase H into the virion environment and this mature form of RNase H bears a conserved N-terminal amino acid residue that makes it a potential substrate for the cellular N-end rule pathway (Figure 3-1). In this study we set out to examine the role of the N-terminal residue of RNase H on the life cycle of HIV-1. To this end we generated

virions containing mutations of the N-terminal RNase H tyrosine residue. We observed that most of the amino acid substitutions led to a complete or almost complete absence of RT in the virion (Figure 3-2). The nature of the amino acid substitution that leads to the absence of detectable RT in the virion was important since mutants that contained structurally similar amino acids to WT (i.e. Trp and Phe) at the N-terminus of RNase H had both subunits of RT at levels similar to WT. Similar observations were reported by another group following introduction of multiple mutations in the p51/RNase H cleavage site region (222). Since that study introduced multiple mutations that led to the degradation of RT in the virus particle, it was not possible to determine which mutation is responsible or what type of amino acids are tolerated at any specific residue. In this study we report on mutagenesis of only the N-terminal residue and our results point to the importance of the nature of the amino acid at the N-terminal amino acid residue of the RNase H for the stability of RT in the virus particle. This is also strongly suggested by the high conservation of the Tyr residue across HIV-1 isolates (Figure 3-1).

In addition to the absence of RT, some RNase H mutants also contained diminished amounts of intravirion integrase (Figure 3-2). There is also a concomitant appearance of peptide around 25 kDa (Figure 3-2 and data not shown). It is probable that a selection of RT mutations lead to a structural change in the RT-Integrase polyprotein that leads to the exposure an alternative protease

cleavage site in integrase. It is important to note that some of the mutant virions didn't contain significant amounts of this possible alternative cleavage product including the proline mutant that has the most drastic effect on RT stability. This may indicate a different or additional mechanism for integrase degradation for these mutants. It is important to note that relative amounts of integrase correlated with the relative amounts of RT found in the RNase H mutants. This may indicate the degradation of a higher molecular weight intermediate induced by the structural change in the polyprotein.

The fact that we observed proteolytic processing of Gag to p24 by viral protease and the presence of at least some amounts of integrase in each mutant combined with the observation that GagPol and Gag is found to be expressed in the RNase H mutants indicate that any presumed deficiency in GagPol production and packaging cannot explain the drastically low levels of RT found in some of the RNase H N-terminal mutants.

Infectivity of the N-terminal RNase H mutants correlated with RT content in the virion. With viruses containing structurally similar amino acids to WT Tyr amino acid at the RNase H N-terminus (Trp and Phe) reporting only a slight decrease in infectivity while the other mutants resulted in complete or almost complete lack of infectivity (Figure 3-3). Even though some mutants retained a limited amount of p51 in the virus particle the presence of p66 is required for the reverse transcription process (248). One of our mutants (Met) showed limited levels of infectivity. A closer look at the immunoblots probed with monoclonal or polyclonal



RT antibody reveals that the Met mutant did contain limited amounts of p66 in the virion that explains the residual level of infectivity observed in this mutant (data not shown).

Despite the degradation of both RT and integrase, we didn't observe a deficiency in intravirion incorporation of GagPol in our RNase H mutants. Moreover appearance of identical proteolytic cleavage patterns in Gag processing indicates that activity of viral protease is not impacted by mutations at the N-terminal amino acid residue of HIV-1 RNase H (Figure 3-4).

The mechanism of the formation of the RT p51/p66 heterodimer is not clearly established. Two possible models have been proposed. In the concerted model, p66 and p51 subunits are separately cleaved out of different GagPol molecules and come together to form the heterodimer. In contrast, the sequential model posits that following the formation of an initial homodimer by two p66 subunits the RNase H domain of one of the subunits is cleaved to form the heterodimer of p66/p51. Even though most of the crystallographic and functional evidence points to the sequential model, definitive evidence is still missing. Since the RT of some our RNase H N-terminal mutants are degraded in the virus particle, we explored the possibility of this degradation affecting a non-mutant RT p66 species by delivering the RT as a GagPol independent vpr fusion protein into the virus.

While these results do not clearly distinguish between the two models of RT heterodimer formation, they indicate that degradation of RT caused by the RNase H N-terminal substitutions is confined to the specific p66 species that contains the mutation. Hence we observed that trans-complementation with WT RT-Integrase vpr fusion protein substantially rescued infectivity in our RNase H mutants.

Proteolytic processing of the precursor polyproteins of HIV-1 is an ordered series of events carried out by the viral protease following the assembly of viral components. A conformational change due to a mutation in the polyprotein may expose alternative cleavage sites leading to additional cleavages of the viral polyprotein and result in loss of infectivity. We tested the possibility that the RNase H N-terminal mutants were degraded by the HIV-1 protease using both an *in vitro* and an intravirion assay. *In vitro* experiments relied on the ability of externally added viral protease to complete the processing of *in vitro* translated GagPol precursor leading to the appearance of mature viral proteins (261). With the caveat that *in vitro* conditions may not exactly represent the conditions inside the virus particle we did not observe any degradation of RT for the proline RNase H mutant (Figure 3-8A). This led us to question the role of HIV-1 protease in the degradation of RT in N-terminal RNase H mutants. An intravirion assay showed the absence of RT p66 species bearing the proline RNase H mutant containing in the virions with the inactivated viral protease (Figure 3-8B) although its

expression in the producer cell was unaffected. However further analysis using co-IP showed that the absence of RT p66 in these virions is caused by the impaired interaction between vpr-pro50-p51 and RNase H proline mutant p66 (Figure 3-8D). This indicates that dimerization of RT subunits is impaired in this RNase H mutant. Previous studies have shown that dimerization of RT subunits is a prerequisite for the activity of the enzyme (267-269). Moreover, mutations in the tryptophan repeat motif of RT has been shown to cause defects in dimerization (268, 269) and these results were later confirmed using the same pLR2p constructs utilized in this study (266). While some of these tryptophan motif mutants also showed decreased levels of RT in the virus particle (268), this degradation is not as drastic as the degradation we observed in RNase H N-terminal mutants. It is possible that the lack of dimerization is a side effect of the structural change brought upon by this mutation, and not the cause of the degradation in the virus particle. Others have speculated that RT-RNase H cleavage site is not involved in the RT dimer formation (270). Here we show evidence that at least one of the amino acids in this cleavage site has a role in association of RT subunits.

It is notable that while the results from our *in vitro* assay indicates that viral protease may not be responsible for the degradation of RNase H proline mutant of RT p66, our vpr complementation and protease inhibitor experiments clearly indicate that release of the mutant RT p66 species from the polyproteins by viral

protease cleavage is required, since GagPol and vpr fused polyproteins were stable even when harboring the unstable RNase H mutations.

Recent advances in proteomic technology have enabled more detailed analysis of the protein content of HIV-1 virus particles. Indeed multiple studies to date have identified cellular proteins packaged into HIV-1 virions (271-274). Relevant to this study the presence of multiple cellular proteases have been reported. However there is no significant overlap in the identity of the proteases identified in these studies. Indeed the only protease that is common to two of these studies is carboxypeptidase D (271, 272). In addition, Santos *et al.* found this protease only in viral particles generated from one of the cell lines they used (272). While this indicates the importance of the specific cell line and methodology used for these studies, we believe that RT degradation of mutants is caused by a protease that is broadly expressed since the same phenomenon (of degradation of RT mutants as result of mutations in this region) was observed when monkey kidney cells (COS-7) and human T cells (MT-2) were used for virus production (222).

Due to its importance as an anti-viral target reverse transcriptase of HIV-1 is one of the most highly studied proteins. Several groups have introduced amino acid substitutions at different subdomains of RT(222, 223, 251, 268, 275-278). Many

of these mutants led to destabilization of the RT protein in the virus particle as well as loss of infectivity. Some studies have concluded that the viral protease is responsible for the degradation of the mutant RT. Nature and the position of the mutation may determine which protease is responsible for RT degradation. However it is important that the potential effect of cellular proteases should not be ignored based on the lack of degradation of GagPol polyprotein in the absence of active viral protease. Our results indicate that liberation of the mutant subunit from the fusion polypeptide is a prerequisite for degradation and previous results should be re-evaluated in this light. This study could have implications for the development of small molecules that distort the structure of the HIV-1 polyprotein in the Pol polypeptide to engage a protease for the destruction of essential viral enzymes.

## **Materials and Methods**

### **Reagents and Cell Culture**

293T and Jurkat cells were obtained from the American Type Culture Collection (ATCC). 293T cells were maintained in Dulbecco's Modified Eagle Medium (Cellgro) supplemented with 10% Fetal Bovine Serum, FBS (Gemini Bioproducts). Jurkat cells were maintained in Iscove's Modified Dulbecco's Medium (ATCC) supplemented with 20% FBS.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; p24 Monoclonal Antibody (183-H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly, HIV-1 HXB2 Integrase Antiserum (aa 23-34) from Dr. Duane P. Grandgenett, HIV-1 RT polyclonal antibody, pEGFP-Vpr (cat# 11386) from Dr. Warner C. Greene and Ritonavir.

Monoclonal antibody for RT (1.149 B6) was previously isolated and characterized(279).

Mouse monoclonal (IgG2a) antibody against HIV-1 vpr was obtained Cosmo Bio USA (Carlsbad, CA).

Secondary p24 antibody for ELISA was collected from a hybridoma cell line obtained from ATCC (HB-9725). Antibody isolation from the hybridoma cell line was performed using standard protocols as described previously (257). Secondary antibody for ELISA; goat-anti-mouse-HRP IgG2A was obtained from Southern Biotech (Birmingham, AL). Goat-anti-mouse-horseradish peroxidase and goat-anti-rabbit-horseradish peroxidase (HRP) secondary antibodies and West Femto enhanced chemiluminescent (ECL) HRP substrate were obtained from Thermo Scientific (Rockford, IL).

## Plasmid Constructs and Mutagenesis

Plasmids were used for VSVg pseudotyped HIV-1 production were: CSII-EGFP; an HIV-1 based vector encoding for GFP driven by EF-1a promoter.  $\Delta$ NRF; encodes for gag, pol, rev, tat and vpu of HIV-1. pMDg; encodes for vesicular stomatitis virus glycoprotein.

RNase H N-terminal mutations were introduced into  $\Delta$ NRF by PCR mutagenesis (PCR Primers available upon request). PCR products which contain the specific mutations were cut with KpnI and ligated back into KpnI digested  $\Delta$ NRF plasmid.

Viral protease inactivating D25A mutation was introduced into  $\Delta$ NRF by overlap PCR (Primers available upon request). Final PCR product was cut with SacII and SbfI, and cloned into  $\Delta$ NRF.

For the vpr complementation assays, a mammalian expression plasmid, pRK5, was used to clone the vpr fusion proteins. Vpr was amplified from pEGFP-Vpr using the following primers; forward; 5' CTCGGATTACCGCCATGGAACAAGCCCCAGAAGAC 3', reverse; 5' CGC GAA GCT TCA GTT CCA GAT CTG AGT AGG ATC TAC TGG CTC CAT TTC

TT 3'. PCR products were digested with BamHI and HindIII and cloned into pRK5. This construct, pRK5-vpr, was used for cloning WT or RNase H mutant RT or RT-Integrase. pRK5-vpr-RT constructs were generated by amplifying RT sequence including the protease cleavage site between Protease and RT from WT or RNase H mutant  $\Delta$ NRF constructs using following primers; Forward 5' GCT CAA GCT TAC TTT AAA TTT TCC CAT TAG TCC 3', Reverse 5' GCT CAA GCT TTT ATA GTA CTT TCC TGA TTC 3'. PCR products were then digested with HindIII and cloned into pRK5-vpr. Same strategy was used for generating pRK5-vpr-RT-IN constructs, the following reverse primer together with forward primer above was used for generating the PCR product including RT and Integrase; 5' GCT CAA GCT TTT AAT CCT CAT CCT GTC TAC 3'.

pLR2P-vpr-p51-IRES-p66 was previously described and was a kind gift from Dr John Kappes (263). RNase H mutants were cloned into this plasmid by first amplifying the  $\Delta$ NRF construct with the corresponding mutation with the following primers; 5' CAG TAA ATT TAA AGC CCG GGA TGG ATG G 3' and 5' GGA TCT CGA GTT ATA GTA CTT TCC TGA T 3'. PCR products were digested with XmaI and XhoI and cloned into pLR2P-vpr-p51-IRES-p66. pLR2P-vpr-pro50-p51-IRES-p66 was generated by first amplifying a region of  $\Delta$ NRF containing 150 nucleotides of viral protease and p51 subunit of RT using the following primers; 5' TAG ATC AGA TCT AAT TGG AGG TTT TAT CAA AGT AG 3' and 5' ATC TAC



ACG CGT TTA GAA AGT TTC TGC TCC TAT 3'. PCR products were digested with BglII and MluI and ligated into pLR2P-vpr-p51-IRES-p66 cut with the same enzymes. pLR2P-vpr-p51 $\Delta$ p66 was generated by first digesting pLR2P-vpr-p51-IRES-p66 with XmaI and XhoI and then re-ligating the plasmid after filling the 5' overhangs generated by the restriction enzymes with T4 DNA polymerase.

pRK5-GagPol was generated by first PCR amplifying GagPol from  $\Delta$ NRF using the following primers; 5' TCG ATT GAA TTC GCC ATG GGT GCG AGA GCG TCG G 3' and 5' GCT CCT GTC GAC TTA ATC CTC ATC CTG TCT 3'. PCR products were digested with EcoRI and SalI and cloned into pRK5. pRK5-GagPol-FS was generated by overlap PCR using following primers: Left pair 5' GGC AAA GAA GGG CAC ACA GCC 3' and 5' CCC TGA GGA AGT TAG CCT GTC TCT CAG TAC 3', right pair 5' GGC TAA CTT CCT CAG GGA AGA TCT GGC CTT CC 3' and 5' GTT GAC AGG TGT AGG TCC TAC 3'. Final product was digested with ApaI and BclI and cloned into pRK5-GagPol. RNase H mutants were cloned into pRK5-GagPol-FS from the  $\Delta$ NRF containing the corresponding mutation using BsrGI digestion.

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### **Virus production and infectivity assays**

HIV-1 vectors were generated by transiently transfecting three plasmids into 293T cells as described previously (231, 232). 15µg of CSII EGFP, 10µg of  $\Delta$ NRF and 5µg of pMDG were transfected using the method of Chen and Okoyama(233). For vpr complementation assays 10 µg of the corresponding vpr fusion protein expressing plasmids were used for transfections. 72 hours after transfection virus was collected and filtered through a 0.45 µM membrane. Filtered virus was concentrated by ultracentrifugation (100,000 x g, 2 hours at 4°C). Viral pellet was resuspended in Phosphate buffer saline (PBS) and aliquots were stored at -80°C. Concentrated virions were normalized to WT virus using p24 ELISA as described previously (257). MOI for WT was determined by infecting  $1 \times 10^5$  Jurkat cells with 10 fold dilutions of the viral preparation. Infections for mutant or vpr complemented viruses were done by using p24 equivalent levels of concentrated viral supernatants (based on p24 ELISA) that correspond to the MOI value for WT virus. 72 hours after the infections EGFP expression was quantified by flow cytometry on a Becton-Dickinson FACScalibur.

### ***In vitro* transcription and translation assays**

Transcription and translation of WT and RNase H mutant pRK5-GagPol-FS was performed using the TNT coupled Reticulocyte Lysate System with SP6 polymerase (Promega) according to the manufacturer's instructions. 4 µL of the

translation product was used for western blotting. For the external HIV-1 protease processing reactions 4  $\mu$ L of translation products were incubated with 1  $\mu$ g of HIV-1 protease (Abcam) in phosphate buffer (25 mM NaCl, 25 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM dithiothreitol and pH 7.0) in 20  $\mu$ L reaction volumes at 30°C for 2 hours. The entire reaction volume was used for western blotting.

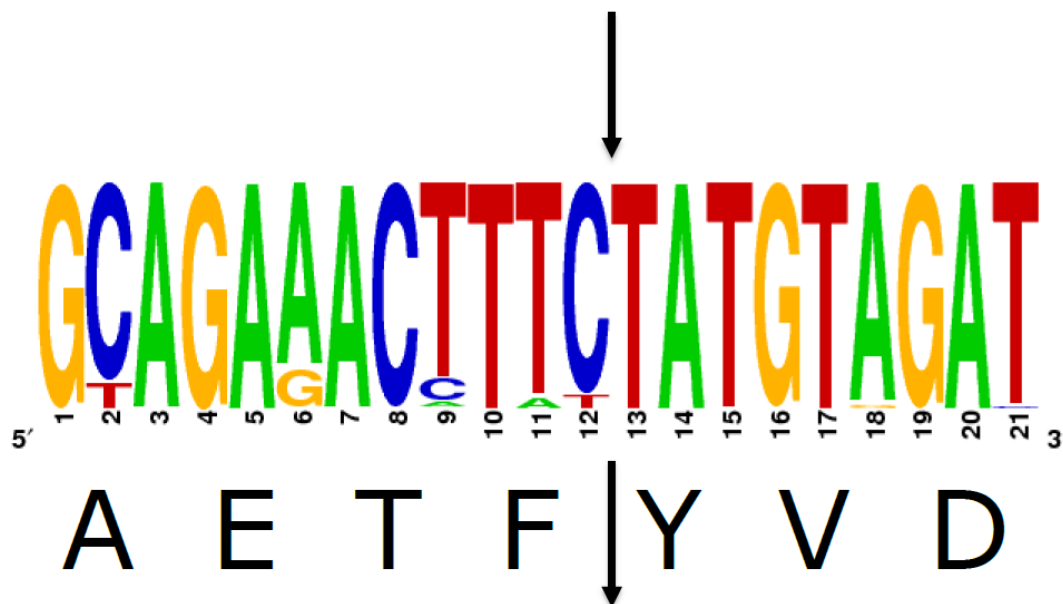
### **Analysis of viral proteins by immunoblotting**

Viral pellets and *in vitro* transcription/translation products were dissolved in loading buffer (0.25M Tris-HCl, pH 6.8, 15% SDS, 50% glycerol, 25%  $\beta$ -mercaptoethanol, 0.01% bromophenol blue) and proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and subjected to immunoblotting using the antibodies indicated.

For the analysis of protein expression from vpr-pro50-p51-IRES-p66,  $5 \times 10^5$  293T cells which were cotransfected with this plasmid together with  $\Delta$ NRF D25A were lysed in cell lysis buffer (20 mM Tris-HCl [pH 8.0], 1 mM  $\text{CaCl}_2$ , 150 mM NaCl, 1% Triton) and proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and analyzed by immunoblotting as described above.

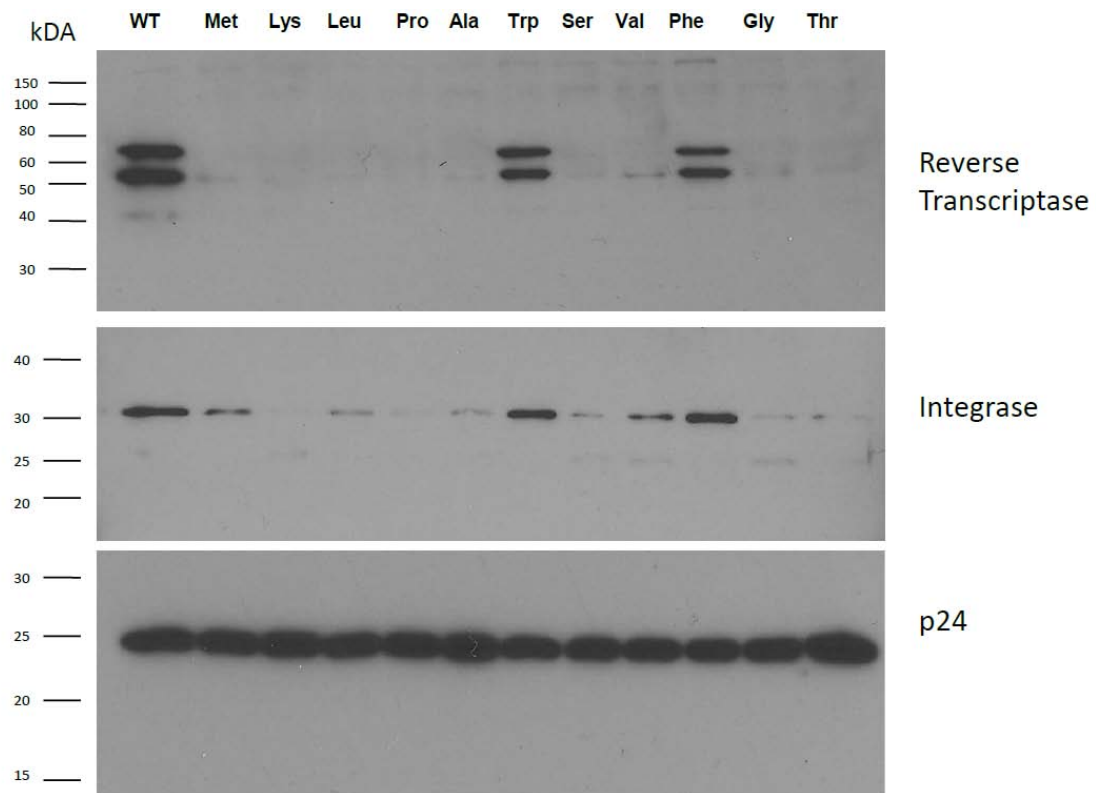
### **Co-Immunoprecipitation (Co-IP) Assay**

293T cells were co-transfected with  $\Delta$ NRF D25A and pLR2P-vpr-p51-IRES-p66wt, pLR2P-vpr-p51-IRES-p66pro or pLR2P-vpr-p51 $\Delta$ p66 as described above. 24 hours after transfection  $5 \times 10^6$  cells were lysed in immunoprecipitation buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton and 0.5% sodium deoxycholate). 5% of this lysate was used as input for the assay. Rest of the lysate was incubated with the Vpr antibody for 1 hour at 4°C. This mixture was then incubated with protein G beads (bio-world) for 2 hours at 4°C. Lysate-bead mixture was washed with the immunoprecipitation buffer 3 times and immunoprecipitated proteins were eluted with loading buffer (0.25M Tris-HCl, pH 6.8, 15% SDS, 50% glycerol, 25%  $\beta$ -mercaptoethanol, 0.01% bromophenol blue). This eluate was used for SDS-PAGE and immunoblotting as described above.

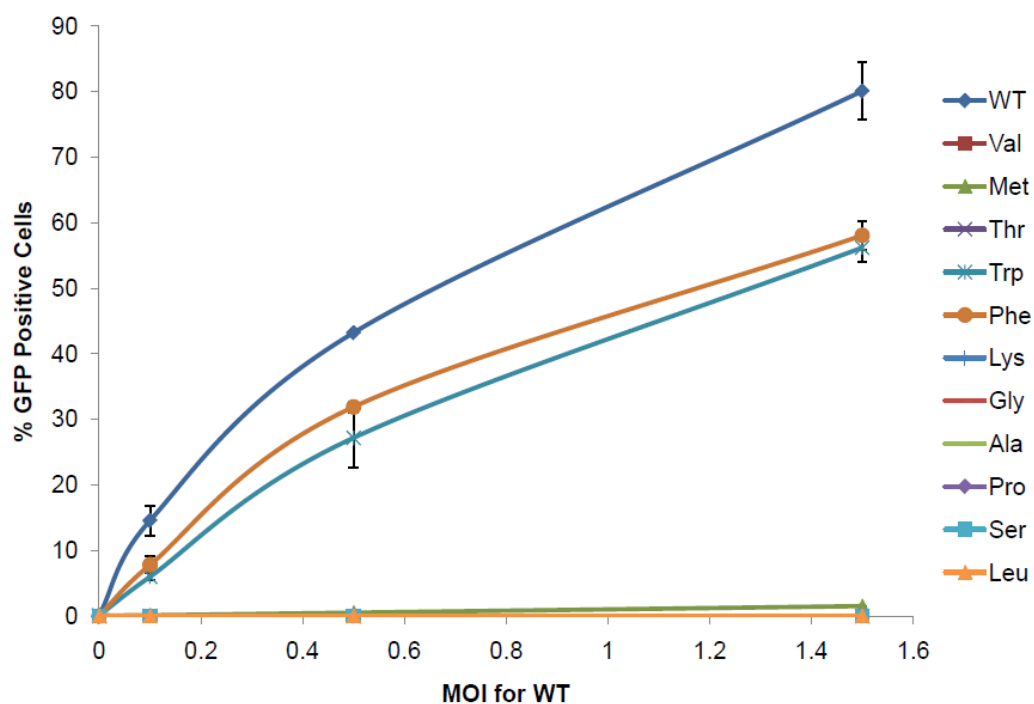


**Figure 3-1. N terminal residue of HIV-1 RNase H is highly conserved.**

Sequence of the protease cleavage site between RT (p51) and RNase H was analyzed for 1850 isolates of HIV-1 and SIVcpz present in Los Alamos HIV Database (<http://www.hiv.lanl.gov/webcite>) using the web alignment tool. Amino acid that corresponds to the conserved sequence is shown at the bottom. The sequence logo at the top was generated using WebLogo (<http://weblogo.berkeley.edu/webcite>).

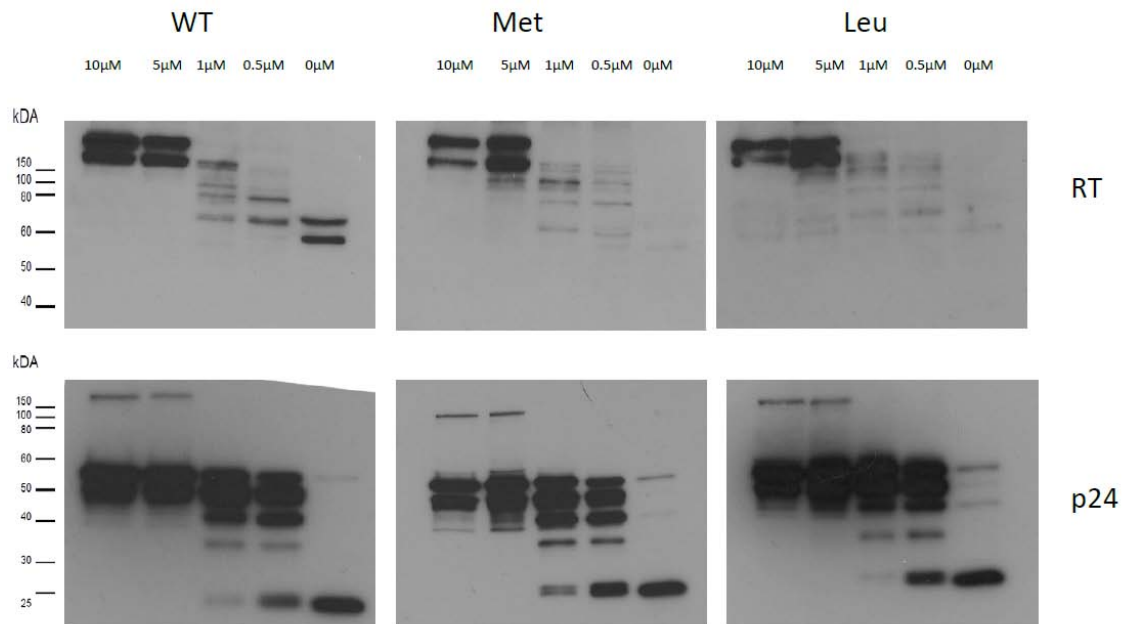


**Figure 3-2. Changing the N terminal residue of HIV-1 RNase H leads to instability of RT and integrase in the virus particle.** Equivalent p24 amounts of WT and RNase H N terminal mutant virions were analyzed by western blotting following production in 293T cells using helper plasmids and concentration by ultracentrifugation. Particles were probed with RT(top), integrase(middle) and p24(bottom) antibodies



**Figure 3-3. Effect of N-terminal RNase H mutations on HIV-1 infectivity.**

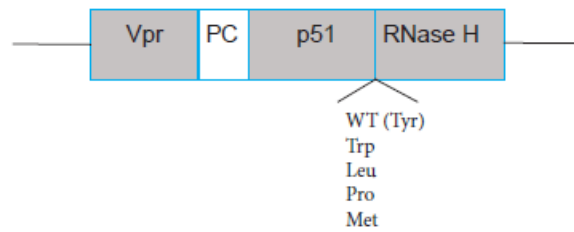
Jurkat cells were infected with p24 equivalent amounts of VSVg pseudotyped WT or mutant RNase H mutant HIV-1 vectors. Infectivity was measured by flow cytometry 3 days post infection. MOI for WT was measured as indicated in the Methods section.



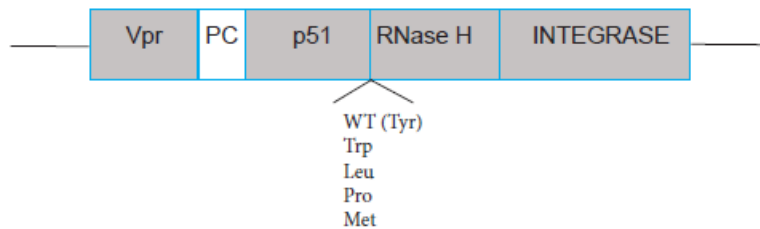
**Figure 3-4. Intravirion processing of Gag and Gag-Pol polyproteins in HIV-1 RNase H N-terminal mutants.** WT and two representatives of RNase H mutant HIV-1 virion particles were produced in the presence of varying concentrations of the HIV-1 protease inhibitor ritonavir and the cleavage pattern of the Gag and Gag Pol polypeptides were analyzed by immunoblot analysis. WT, as well as methionine (Met) and Leucine (Leu) RNase H mutants were probed with antibodies to RT (top) or p24 (bottom).



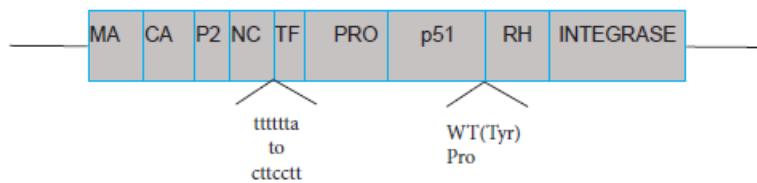
A. pRK5-Vpr-RT



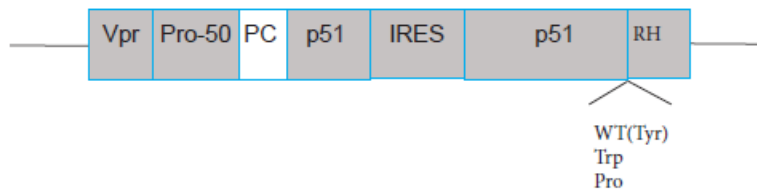
B. pRK5-Vpr-RT-IN



C. pRK5-GagPol-FS

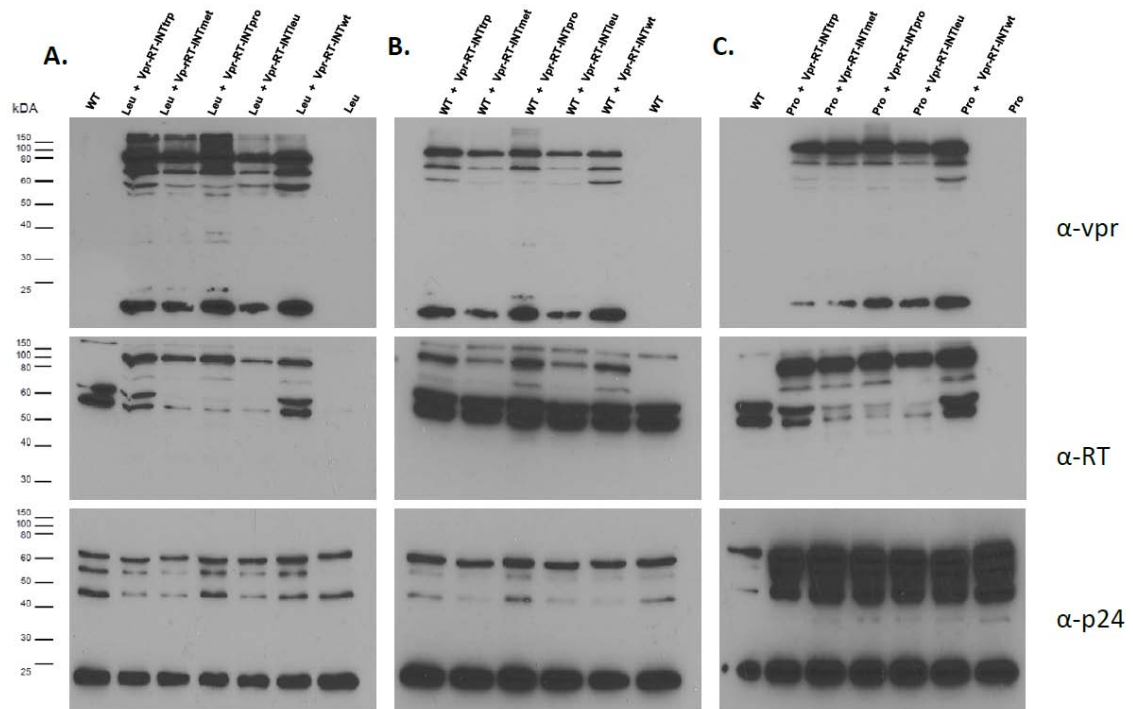


D. pLR2P-vpr-pro50-p51-IRES-p66

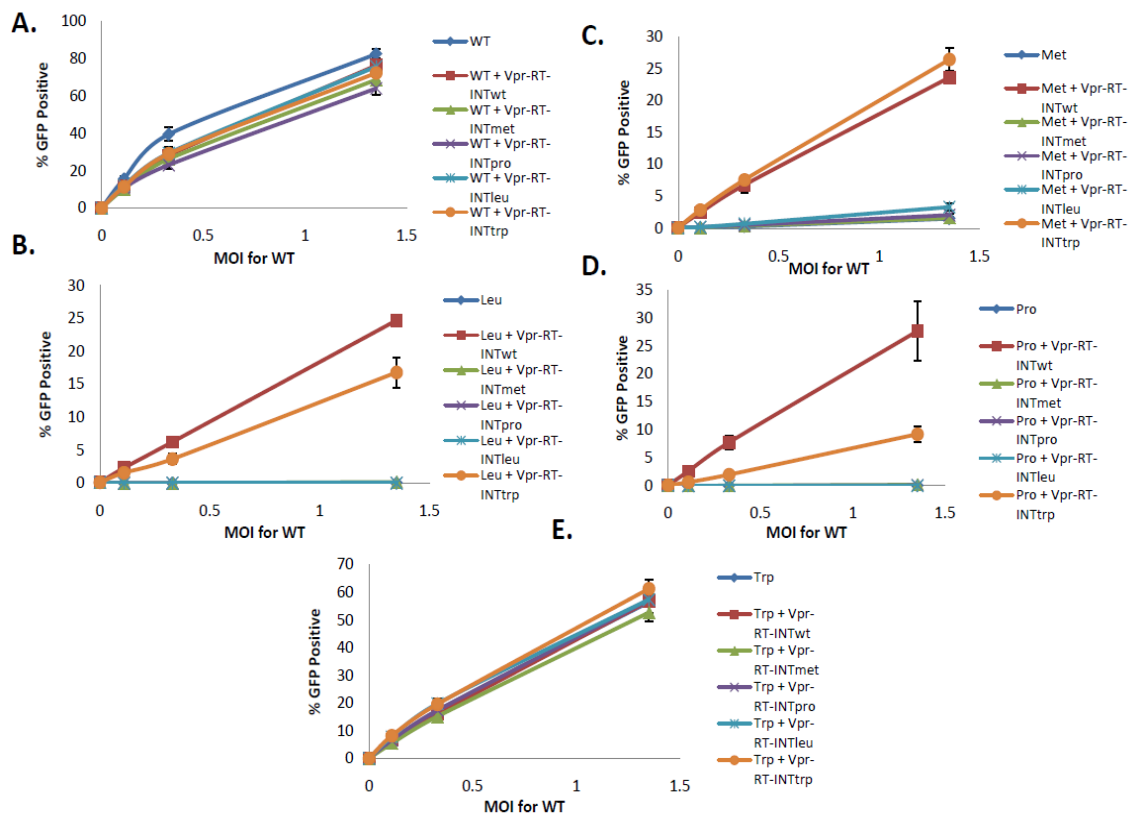


**Figure 3-5. Constructs used for Vpr complementation and *in-vitro* studies.**

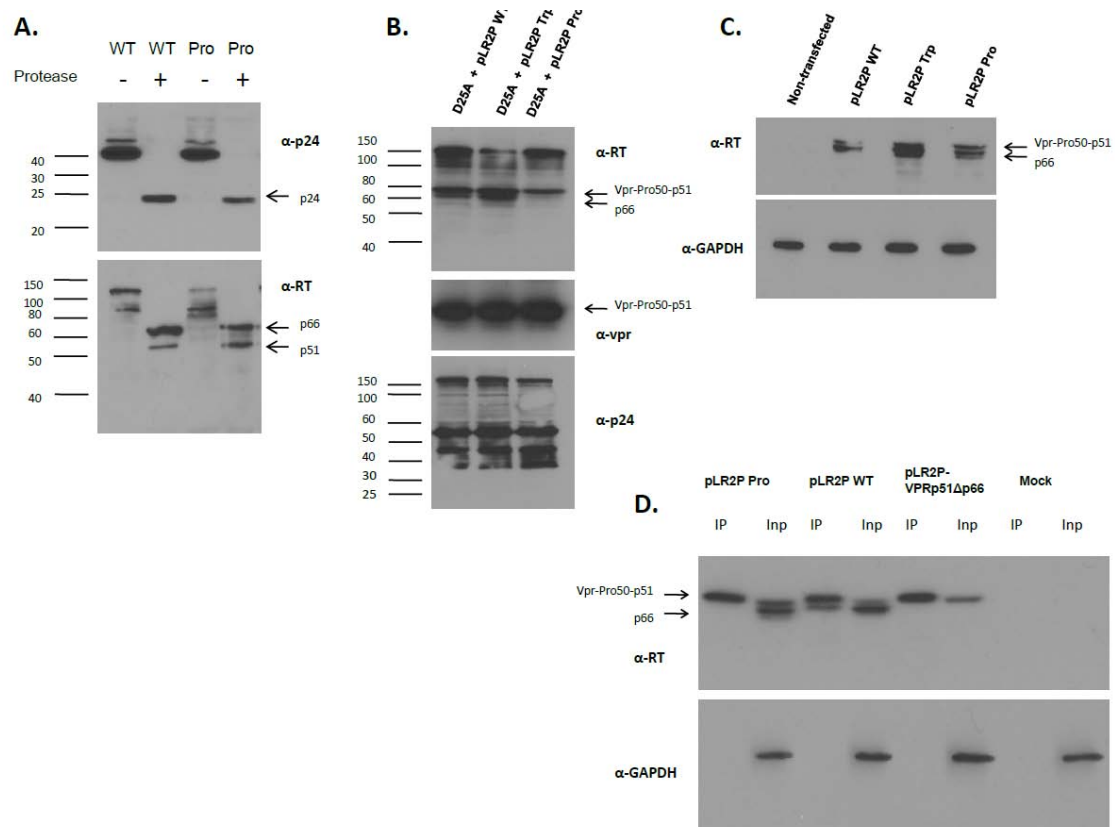
Position and identity of RNase H N-terminal substitutions are indicated. Frameshift causing mutation for pRK5-GagPol-FS is shown. PC: protease cleavage site between viral protease and RT.



**Figure 3-6. Analysis of Vpr-RT-Integrase complemented HIV-1 virions.** HIV-1 virions with GagPol containing WT (B) and RNase H N-terminal mutants Leu(A) or Pro (C) were produced in 293T cells with helper plasmids in the presence or absence of expression plasmids encoding for Vpr-RT-Integrase fusion proteins with WT or N-terminal RNase H mutant RT. Concentrated virions were normalized for p24 with ELISA and analyzed by western blotting using vpr (top), RT(middle), p24(bottom) antibodies.



**Figure 3-7. Infectivity of Vpr-RT-Integrase complemented WT or RNase H N-terminal mutant viruses.** Jurkat cells were infected with p24 equivalent WT (A) or N-terminal RNase H Leu (B), Met (C), Pro (D), Trp (E) mutant VSVg pseudotyped HIV-EGFP containing WT or RNase H mutant vpr-RT-IN fusion proteins. Infectivity was determined by flow cytometry 3 days post infection. MOI for WT was measured as described in Methods.



**Figure 3-8. *In vitro* and intravirion analysis of the role of viral protease in the degradation of RNase H mutants.** **A.** pRK5-GagPol-FS was used for *in vitro* transcription translation by RRL. Translation products were incubated at 30°C for 2h with HIV-1 protease or phosphate buffer and analyzed via immunoblotting. **B.** Protease inactivating D25A mutant virions were produced in 293T cells in the presence of vpr-pro50-p51/p66 plasmids encoding WT or RNase H mutant p66. Concentrated virions were analyzed by western blotting using the indicated antibodies following normalization via p24 ELISA. **C.** 293T cells were co-transfected with vpr-pro-p51/p66 plasmids encoding WT or RNase

H mutant p66 and  $\Delta$ NRF D25A. Cellular extracts were analyzed by immunoblotting. **D.** 293T cells were co-transfected with vpr-pro-p51/p66 plasmids encoding WT or RNase H mutant p66 and  $\Delta$ NRF D25A. Co-IP was performed on cell extracts using vpr antibody and protein G beads. Arrows indicate the identity of the RT, p24 and vpr antibody detected fragments. Antibodies used for each blot is indicated in bold with an  $\alpha$  sign.

**Table 3-1. HIV-1 RNase H N-terminal mutants**

<b>Cleavage site (RT/RH)</b>	<b>N-end Rule Designation</b>	<b>Amino acid at P1'</b>	<b>Reason for selection</b>
<b>AETF /<u>Y</u>VD (WT)</b>	<b>Destabilizing</b>	<b>Tyrosine</b>	<b>WT</b>
<b>AETF /<u>M</u>VD</b>	<b>Stabilizing</b>	<b>Methionine</b>	<b>N-end rule</b>
<b>AETF /<u>F</u>VD</b>	<b>Destabilizing</b>	<b>Phenylalanine</b>	<b>Structurally conserved</b>
<b>AETF /<u>W</u>VD</b>	<b>Destabilizing</b>	<b>Tryptophan</b>	<b>Structurally conserved</b>
<b>AETF /<u>T</u>VD</b>	<b>Stabilizing</b>	<b>Threonine</b>	<b>N-end rule</b>
<b>AETF /<u>L</u>VD</b>	<b>Destabilizing</b>	<b>Leucine</b>	<b>N-end rule</b>
<b>AETF /<u>K</u>VD</b>	<b>Destabilizing</b>	<b>Lysine</b>	<b>Long basic side chain</b>
<b>AETF /<u>A</u>VD</b>	<b>Stabilizing</b>	<b>Alanine</b>	<b>N-end rule</b>
<b>AETF /<u>P</u>VD</b>	<b>Stabilizing</b>	<b>Proline</b>	<b>Drastic structure change</b>
<b>AETF /<u>G</u>VD</b>	<b>Stabilizing</b>	<b>Glycine</b>	<b>Small side chain</b>
<b>AETF /<u>S</u>VD</b>	<b>Stabilizing</b>	<b>Serine</b>	<b>N-end rule</b>
<b>AETF /<u>V</u>VD</b>	<b>Stabilizing</b>	<b>Valine</b>	<b>Small hydrophobic side chain</b>

## **Chapter 4**

# **Characterization of Resistance to Retroviruses and Retroviral Vectors in a Human Myeloid Cell Line**

## **Summary**

Retroviruses interact with various supportive and restrictive factors in the host cell throughout their replication cycle. Recent years have seen an increase in the efforts to identify host cell factors involved in retroviral infection. Cell lines that are non-permissive to retroviral infection have been particularly useful in discovering major host cell proteins involved in viral life cycle. Here we describe characterization of a human myeloid leukemia cell line, KG-1, that is resistant to infection by retroviruses and retroviral vectors. We show that KG-1 cells are completely resistant to infection by Vesicular Stomatitis Virus Glycoprotein (VSVG) pseudotyped retroviruses as well as VSV due to a defect in VSVG binding. Moreover our results indicate that entry by xenotropic retroviral envelope glycoprotein RD114 is impaired in KG-1 cells. Finally we also discovered a post-entry block at early phase of the retroviral life cycle in KG-1 cells.



## **Introduction**

As obligate intracellular pathogens retroviruses are intimately dependent on host cell factors throughout their life cycle. Since the viral genome only encodes a limited number of genes, retroviruses need to utilize various functions of the host cell machinery to complete their replication. In addition to the factors that are exploited by retroviruses (called cofactors), several cellular proteins have been found to act in an inhibitory role against retroviral infection. These are called restriction factors. Various methods have been used to identify retroviral cofactors and restriction factors including genomewide RNAi screening (49-51), analyses of host proteins that interact with specific viral proteins (21, 97) and characterization of cells refractory to viral infection (23, 130).

Our laboratory as well as other groups previously isolated mammalian cell lines resistant to infection by retroviruses using different methods (229, 280). Characterization of some of these cell lines led to identification of multiple host factors that are involved in retroviral infection such as Zinc Finger Antiviral Protein and fasciculation elongation protein zeta-1 (281, 282). Moreover, one of the HIV-1 co-receptors, CXCR4, was identified by cDNA library complementation of non-permissive cells (30). Collectively these and similar studies indicate the usefulness of non-permissive cells in understanding retrovirus-host interactions.

Vesicular Stomatitis Virus (VSV) is an enveloped virus with a negative stranded RNA genome and is a member of the family Rhabdoviridae. Despite causing only mild disease in humans, VSV has been studied extensively due to its potential as an oncolytic agent and the use of its envelope glycoprotein (VSVG) to alter tropism of retroviral vectors (283-286). Broad tropism of VSV makes these VSVG pseudotyped retroviruses excellent gene delivery tools. This remarkable broad tropism of VSV indicates that the receptor for VSVG mediated entry must be ubiquitously present in all the widely differing cell types from different species that have been transduced. Prompted by this observation, several studies have suggested that plasma membrane lipids such as phosphatidyl serine or gangliosides can serve as the cellular receptors of VSV (287-289). However, subsequently Coil and Miller showed more direct evidence that phosphatidyl serine is not the receptor for VSV even though it may be involved in a later step following receptor mediated endocytosis (290).

Two recently published studies have increased our understanding of VSVG mediated binding to cells. Bloor et. al. have shown that gp96, a ubiquitous endoplasmic reticulum chaperone, can rescue VSVG binding deficiency in a mouse B cell line that was generated by chemical mutagenesis. Based on this observation the authors propose that gp96 is either directly interacting with the VSVG receptor or is required for the synthesis of the functional receptor (291). In a more recent study, Finkelshtein et al. showed that low density lipoprotein (LDL)

receptor functions as the major entry receptor for VSV, while the other members of the LDL receptor family are used as the alternate receptors (292).

In this study we investigated the resistance to retrovirus and VSV infection in a lymphomyeloid progenitor cell line called KG-1 cells. A study in late 80s have demonstrated that these cells are refractory to transduction by a Murine Leukemia Virus (MLV) based vector (293). Here we show that KG-1 cells are impaired in binding VSV and VSVG pseudotyped retroviruses. Our results also indicate that the presence of functional LDL receptor family members is not sufficient for VSVG binding. We further demonstrate that retroviruses pseudotyped with a xenotropic retroviral envelope glycoprotein, RD114, are impaired in their entry to KG-1 cells. Finally our results indicate that post entry KG-1 cells are refractory to infections by retroviruses.

## **Results**

### **KG-1 cells are resistant to infection by HIV-VSVG and MLV-VSVG**

During gene transfer experiments in our laboratory, we observed differential efficiency in infectivity of different cell lines by VSVG pseudotyped HIV-1 (HIV-VSVG) based vectors. Figure 4-1 illustrates this for GFP encoding HIV-VSVG in 6 different human cell lines. While some cell lines (such as a T cell line, Cem-A)

showed substantially lower levels of transduction efficiency than others we observed no GFP positive cells in the KG-1 population (Figure 4-1). Since non-permissive cell lines can be powerful tools to understand virus-host interactions we decided to characterize the block to HIV-VSVG infection further in KG-1 cells. First we infected KG-1 cells with HIV-VSVG at higher multiplicities to test whether the block to infection can be overcome which can be indicative of a restriction factor that can be saturated. As shown in Figure 4-2A, even at multiplicity of infection (MOI) of 10 we didn't observe any transduction in KG-1 cells. To test whether this block is unique to HIV-1 we used another retrovirus; Murine Leukemia Virus (MLV). Again we observed no infection in KG-1 cells incubated with GFP encoding VSVG pseudotyped MLV (MLV-VSVG) at an MOI of 10 (Figure 4-2A). These results indicate that KG-1 cells are resistant to VSVG pseudotyped retrovirus infection. Absolute resistance to retroviruses in KG-1 cells raised the possibility of a general block to viral infection in KG-1 cells. To test this we infected these cells with a virus from a different family. To this end we utilized measles virus which is a negative stranded RNA virus of the paramyxovirus family. We infected Jurkat and KG-1 cells with measles virus encoding a GFP gene and scored GFP positive cells 48 hours after infection. As shown in Figure 4-2B, KG-1 were infected with measles virus approximately 50% less efficiently than Jurkat cells at an MOI of 3. Therefore the block to retroviral infection we observed in KG-1 cells is not caused by a general resistance to virus infection.

## **HIV-VSVG infection is impaired at or before reverse transcription in KG-1 cells**

Next, we determined which stage of the HIV-1 life cycle is impaired in KG-1 cells. We first isolated total DNA from Jurkat and KG-1 cells at different time points following infection with HIV-VSVG and tracked the presence of reverse transcription products via quantitative PCR (qPCR) using different primer sets. We also isolated DNA from cells that were cultured for 6 weeks after infection with HIV-VSVG and tested the presence of integrated provirus in KG-1 and Jurkat cells. As shown in Figure 4-2C we detected no HIV-1 proviral DNA in KG-1 cells infected with HIV-VSVG. This confirms our initial observation using EGFP reporter. Similarly we detected very low levels of 2LTR circles (at least 2 logs lower than Jurkat cells) in KG-1 cells as compared to Jurkat cells at 48 hours post infection (Figure 4-2D). While 2LTR circles serve as surrogate markers for defective integration, substantially lower numbers such as the ones we observe in KG-1 cells compared to infectable Jurkat cells indicate a decrease in viral cDNA that reaches the nucleus. We also found low levels of early reverse transcription (first jump) products 8 hours after infection in KG-1 cells compared to Jurkat cells (Figure 4-2E). It is important to note that the levels of first jump products detected in KG-1 cells were similar to what was obtained with non-

infected cells. These results indicate the presence of a major block to HIV-VSVG infection at or before reverse transcription followed possibly by another block at a later stage.

### **KG-1 cells cannot be infected by VSV due to a defect in VSVG binding**

Since our qPCR experiments didn't allow us to determine at which point before reverse transcription retrovirus infection was blocked in KG-1 cells we decided to further characterize viral entry via VSVG in these cells. To test this, we determined if infection by VSV is blocked in KG-1 cells. To this end we utilized a virus called VSV-eGFP (a replication competent VSV) that contains the VSV genome encoding an eGFP coding sequence as an extra gene between G and L gene junctions. We infected KG-1 and Jurkat cells with VSV-eGFP at different multiplicities and tracked GFP expression using flow cytometry at different time points (data not shown). Figure 4-3A shows the result of a VSV-eGFP infection at an MOI of 50 with GFP expression measured at 24 hours post infection. We observed no GFP expression in KG-1 cells infected with VSV-eGFP (Figure 4-3A). These results combined with the results obtained using VSVG pseudotyped retroviruses indicate that there may be a block to VSVG mediated entry in KG-1 cells. To test this possibility further, we examined entry of VSVG pseudotyped retroviruses to KG-1 cells. KG-1 and Jurkat cells were incubated with HIV-VSVG at 4°C for 1 hour and washed to remove unbound viral particles. Cells were then

moved to 37°C to initiate entry and start the infection. At different time points post infection cells were collected, washed and treated with trypsin to remove surface bound virions. Cells were then lysed and analyzed by immunoblotting for the presence of HIV-1 capsid protein (p24) as an indicator of viral entry. As shown in Figure 4-3B, while we detected increasing levels of p24 in Jurkat cells over time, we didn't detect any p24 in KG-1 cells. This clearly indicates a defect in entry of HIV-VSVG to KG-1 cells. Next we determined whether VSVG binding is affected in these cells. Using the same assay as above we infected KG-1 and Jurkat cells with HIV-VSVG. However the cells were not treated with trypsin and instead washed extensively with cold PBS after collection at different time points post infection. We reasoned that any bound virus that had not been internalized would still be detected. As shown in Figure 4-3C, presence of p24 is detected in Jurkat cells even when cells were kept at 4°C (0h time point). In contrast we didn't detect any p24 in KG-1 cells at any point after infection (Figure 4-3C). These results indicate a VSVG binding defect in KG-1 cells. Next we validated this result using a different assay. KG-1 and Jurkat cells were incubated with HIV-VSVG at 4°C for 1 hour. Cells were then washed extensively with cold PBS and the presence of bound VSVG pseudotyped virus on the cell surface was assayed using a primary antibody against VSVG and secondary antibody containing a fluorescent tag (Alexa 488). Fluorescence was scored using flow cytometry. As shown in Figure 4-3D and E, while bound VSVG is clearly detected in Jurkat cells (Figure 4-3E), no shift in fluorescence and hence no bound VSVG is detected in

KG-1 cells (Figure 4-3D). All these results collectively indicate that VSVG binding is impaired in KG-1 cells.

### **Functional LDL uptake in KG-1 cells**

While our results so far suggests that KG-1 cells have a defect in VSVG binding, another group has previously generated a Murine cell line through chemical mutagenesis that was found to be impaired in VSVG binding (291). The results of that study suggests that the endoplasmic reticulum chaperone gp96 may be required for VSVG binding. To test whether the absence of gp96 can explain the VSVG binding defect in KG-1 cells we probed KG-1 and Jurkat cell lysates for the presence of gp96 via immunoblotting. We found that gp96 is expressed in KG-1 cells at comparable levels to Jurkat cells (Figure 4-4A). Hence gp96 deficiency doesn't explain the defect on VSVG binding in KG-1 cells.

The identity of the receptor for VSVG has remained a mystery until recently despite the widespread usage of this glycoprotein in gene transfer experiments and the broad tropism of VSV. Results of a recent study suggests that LDL receptor family acts as the receptor for VSVG mediated entry (292). Since our results suggests that KG-1 cells are deficient in VSVG binding, we tested whether functional LDL receptor family members are present on KG-1 cells. For this we utilized an LDL uptake assay using fluorescently labeled LDL (LDL-Dil) (294). To our surprise, KG-1 cells showed robust LDL uptake at levels



comparable to control Jurkat cells (Figure 4-4B and C). We observed the same result when we performed this assay using different concentrations of Dil-LDL and various incubation times (data not shown). This result indicates that KG-1 cells contain functional LDL receptor family members and that the presence of LDL receptor family members is not sufficient for VSVG binding.

### **Entry of RD114 pseudotyped retroviruses is impaired in KG-1 cells**

Our results above did not allow us to conclude whether there is a block to retroviral life cycle in KG-1 cells since the resistance we observed for HIV-VSVG and MLV-VSVG seems to be caused by a deficiency in VSVG binding (Figure 4-2). To test whether KG-1 cells are infectable by retroviruses we used a different envelope glycoprotein to mediate the entry of our retroviral vectors. To avoid any blocks in viral binding we limited our search to envelope proteins with known receptors that are expressed in KG-1 cells. RD114, the envelope glycoprotein of a feline endogenous retrovirus fit this criteria (295, 296). The receptor for this glycoprotein was characterized as a neutral amino acid transporter called SLC1A5 (297). A search on the GEO profiles database (298) also revealed that this gene is expressed in KG-1 cells (GEO accession GDS2251, (299)) . Therefore we produced RD114 pseudotyped GFP encoding HIV-1 and MLV vectors and tested the infectivity of these vectors in KG-1 and Jurkat cells. As

shown in Figure 4-5A, similar to the VSVG pseudotyped vectors we observed no infectivity in KG-1 cells using RD114 pseudotyped HIV-1 and MLV while Jurkat cells were infected at high levels with HIV-GFP (85%) and MLV-GFP (55%) at the titers used. We performed qPCR assays to pinpoint the position of this block. Similar to VSVG pseudotyped vectors we observed background levels of early RT (First Jump) products (Figure 4-5B), and the absence of 2LTR circles (Figure 4-5C) in KG-1 cells infected with RD114 pseudotyped HIV-GFP. To test whether the RD114 pseudotyped HIV-1 can enter KG-1 cells, KG-1 and Jurkat cells were incubated with the virus at 4°C for 1 hour and infection was initiated by moving the cells to 37°C. 2 hours post infection cells were collected and either washed extensively with PBS or treated with trypsin to remove particles bound outside the cell. Cell lysates were analyzed by immunoblotting for the presence of p24. In contrast to the results obtained with VSVG pseudotyped virus, we detected the presence of virus in the absence of trypsin treatment (Figure 4-5D, KG-1, lane labeled no trypsin). However we did not detect any viral capsid protein after trypsin treatment (Figure 4-5D, KG-1, lane labeled trypsin). These results indicate that RD114 pseudotyped HIV-1 vector can bind but cannot enter KG-1 cells.

## **KG-1 cells are refractory to retroviruses pseudotyped with amphotropic envelope**

Lack of entry observed for the RD114 pseudotyped retroviruses prompted us to search for another retroviral envelope glycoprotein with a known receptor. We decided to use amphotropic MLV envelopes 4070A and 10A1 which utilize sodium phosphate co-transporters Pit1 and Pit2 for entry (300). Moreover 4070A enveloped MLV has been previously used to study infectivity of MLV in KG-1 cells (293). Notably viruses pseudotyped with the amphotropic envelopes had very low titers in our hands. While titers were increased after concentration, to test infectivity of KG-1 cells at high enough viral titers we decided to utilize spinoculation for the infection assays with amphotropic envelopes. As shown in Figure 4-6A, KG-1 cells showed low levels of infectivity (between 20-30 fold) compared to Jurkat cells after spinoculation with 4070A pseudotyped MLV over a range of MOIs. Similar results were obtained after spinoculation with 4070A pseudotyped HIV-1, but infectivity difference between Jurkat and KG-1 cells was less (6-8 fold) (Figure 4-6B). We obtained similar results using 10A1 envelope pseudotyped viruses (data not shown). While the levels of GFP positive cells were lower, infectivity difference observed between KG-1 and Jurkat cells were comparable when these cells were infected without spinoculation (data not shown). Similar results were also observed when a different reporter (Ds-Red) and a different promoter driving the reporter expression (CMV) in the vector were used (data not shown). To better pinpoint the stage in the HIV-1 life cycle that is

impaired in KG-1 cells we performed qPCR experiments as described above. As shown in Figure 4-6C, levels of early RT (first jump) products were similar between KG-1 and Jurkat cells at 8 hours post infection. This indicates not only similar levels of reverse transcription progression but also similar levels of viral entry in Jurkat and KG-1 cells. In contrast to the early RT products, we observed 8-10 fold difference in the amount of 2LTR circles at 48 hours post infection between KG-1 and Jurkat cells (Figure 4-6D). While 2LTR circles are considered to be dead end products which is the result of the ligation of the linear reverse transcription product, they serve as surrogate readout of nuclear import. Hence these results indicate a defect in late reverse transcription or nuclear import in KG-1 cells. To test whether the difference observed in the number of GFP positive cells following 4070A pseudotyped HIV-1 infection in KG-1 and Jurkat cells is reflective of the difference in the amount of integrated provirus, we cultured these cells for 4 weeks after infection. The continued culture of the cells will eliminate un-integrated products by dilution and degradation. As shown in Figure 4-6E, there is a 6-8 fold difference in the levels of integrated provirus between KG-1 and Jurkat cells. These observations are consistent with the results obtained for the functional GFP positive cells assay 3 days post infection (Figure 4-6A) and 4 weeks post infection (Figure 4-7A). The difference in the number of GFP positive cells between Jurkat and KG-1 cells after infection with 4070A pseudotyped MLV-GFP is also maintained when the cells were cultured long term (Figure 4-7B).

Accessory factors encoded by lentiviruses have been shown to be crucial in the infection of certain cell types (145, 160). The helper plasmid we use in this study does not encode for vpr, nef and vif proteins. To test whether these accessory factors can alleviate the block observed in KG-1 cells we utilized a vector that contains all the accessory factors. As shown in Figure 4-7C we observed no difference in the relative infectivity of KG-1 cells as compared to Jurkat cells using this vector when compared to the vector that lacked some of the accessory factors (Figure 4-7A). We conclude that the resistance to infection in KG-1 cells cannot be alleviated by the presence of HIV- accessory factors.

## **Discussion**

In this study we characterized the resistance to retroviral and VSV infection in a cell line that was previously shown to be partially refractory to MLV based vectors (293). Characterization of non-permissive cells - either generated by mutagenesis or naturally occurring - to retroviral infection has been a powerful tool for discovering host proteins involved in viral life cycle in the past two decades including two of the most studied restriction factors, APOBEC3G and TRIM5 $\alpha$  as well as the HIV-1 co-receptor CXCR4 (23, 30, 130). The non-permissive cell line we used in this study, KG-1 cells, were isolated from a bone marrow aspirate of a patient with acute myelogenous leukemia (301). Interestingly KG-1 cells show characteristics of myeloid progenitor cells, and are sometimes described as myeloblasts (302, 303). Moreover they express one of

the markers of bone marrow derived progenitor cells, CD34 (293). Previous studies have shown the differentiation potential of KG-1 cells to mature dendritic cells indicating the immature characteristics of these cells (302). These properties of KG-1 cells made them a frequent research tool when studying myeloid cancers and gene transfer into hematopoietic progenitor cells. Our experience with KG-1 cells began when we were studying gene transfer efficiency by lentiviral vectors to different hematopoietic cell lines. We observed an absolute resistance to both HIV-VSVG and MLV-VSVG even at high multiplicities and decided to investigate it further. Analysis of the accumulation of viral cDNA products indicated that the block to infection is at the level of early reverse transcription or at an earlier step. This result guided us to identify the lack of VSVG mediated binding as the reason for the inability of VSVG pseudotyped retroviruses and VSV to infect KG-1 cells. Biochemical and fluorescence based assays we performed to reach this conclusion relied on the binding of the virus particles to the receptors on the cell surface at 4°C. It may be argued that in KG-1 cells binding of VSVG to its cellular receptor is too weak to detect at this temperature. Notably we still didn't detect any virus particles inside or bound outside the cells even when the cells were incubated at 37°C for KG-1 cells in contrast to Jurkat cells.

To our knowledge KG-1 cells represent the first human cell line that is deficient in VSVG binding and entry. Previously a mouse B cell line had been characterized to be deficient for VSVG binding following mutagenesis with a chemical mutagen,

ICR191 (291). The authors of that study identified a frameshift in the gene encoding for the heat shock protein gp96. Complementation with a functional gene for gp96 restored VSVG mediated binding and entry by retroviral vectors (291). As a follow up to this study, the same group demonstrated using plasma membrane profiling that cell surface expression of some of the LDL receptor family members was decreased in the cells lacking gp96 (304). Notably neither of these studies confirmed these results on different cells by depletion of gp96. We note an inconsistency in the data of that study since one clone that didn't express gp96 showed low level infectivity by VSVG pseudotyped MLV vector (291). Our results indicate that KG-1 cells express gp96 and we conclude that this is not the mechanism for the VSVG binding defect in KG-1 cells.

Another group published a different study identifying the LDL receptor family members as the cellular receptors of VSVG (292). This work relies on the observation that soluble LDL receptors can bind VSVG and inhibit plaque formation by VSV and exogenous addition of LDL receptor to LDL receptor deficient human fibroblasts increased infection by VSVG but not LCMV pseudotyped retroviruses. However since LDL receptor deficient cells can still be infected by VSV it was hypothesized that other members of the LDL receptor family can be utilized by VSVG for binding and entry (292). We tested this in VSVG binding deficient KG-1 cells using a fluorescent LDL uptake assay. Our results indicate that KG-1 cells are capable of functional LDL uptake. Even

though our experimental system doesn't allow us to determine whether LDL binding and uptake kinetics is different between KG-1 and Jurkat cells, we can conclude that functional LDL receptor family members are present in KG-1 cells. This result doesn't necessarily exclude the possibility that LDL receptor family members are required for VSVG binding. It is possible that presence LDL receptor family members on the cell surface is simply not sufficient for VSVG binding and entry. Several viruses require more than one entry factor on the cell (28, 29, 305, 306). It is conceivable that VSVG binding requires another protein which may be missing or abnormal in KG-1 cells. Another possibility is that KG-1 cells express a factor that prevents surface expression or modification of the VSVG receptor. Our preliminary studies with cell fusion indicates that this block to VSVG binding may be dominant (data not shown).

Interestingly the envelope protein we chose (RD114) to test retroviral infectivity in KG-1 cells following the observation of VSVG binding defect also failed to facilitate viral entry. Receptor for this envelope glycoprotein was discovered via cDNA complementation of NIH3T3 cells which do not support infection with RD114 unless treated with tunicamycin, which inhibits N-linked glycosylation of proteins (297). In our hands, Tunicamycin treatment of KG-1 cells didn't have any effect to RD114 mediated retroviral entry (data not shown). Our results suggest that RD114 mediated entry but not binding is blocked in KG-1 cells. It has been previously shown that RD114 pseudotyped viruses enter the cells via a pH



independent path (307). While this suggests that fusion of viral and cellular membrane may be happening at the cell surface, there is no detailed study of RD114 mediated viral entry. It has recently been shown that cellular entry of HIV-1 involves receptor mediated endocytosis and the fusion happens at the endosomes in some cell types despite the lack of requirement for pH change (32). A similar mechanism of entry may be used for RD114 mediated entry. It is conceivable that endocytosis through the RD114 receptor is impaired in KG-1 cells. It is also possible that RD114 entry requires more than one receptor and this co-receptor is missing in KG-1 cells. Further experimentation is needed to differentiate between these and other possible mechanism of inhibition of RD114 mediated viral entry in KG-1 cells.

Retroviral infection was previously tested in KG-1 cells by using an MLV based vector pseudotyped with amphotropic 4070A envelope glycoprotein (293). In the aforementioned study analysis of viral cDNA by southern blotting in the infected cells revealed the absence of integrated provirus while the presence of viral cDNA was detected using Hirt extraction of low molecular weight DNA molecules in the cells. Our results contradict with this observation as we observed integrated provirus through GFP expression in KG-1 cells cultured for up to 6 weeks after transduction with MLV pseudotyped with the same amphotropic envelope. We observed similar results with HIV-1 and also observed the presence of proviral DNA in KG-1 cells via qPCR analysis. Discrepancy between

our results and the previous observation could be caused by the difference in sensitivity of the assays. Since even with spinoculation we observed very low levels of MLV infectivity in KG-1 cells, it is possible that integrated provirus may not be detectable by southern blot at low multiplicities. Moreover our qPCR results indicate that HIV-1 infection in KG-1 cells is impaired at the nuclear import or late reverse transcription step. This means that reverse transcription products are found in substantially higher amounts in KG-1 cells than the integrated proviral DNA. If a similar mechanism acts on MLV infection unintegrated viral DNA may be detected with better sensitivity than integrated provirus.

There are three major restriction factors identified so far that affect the early phase of the HIV-1 life cycle; APOBEC3G, Trim5 $\alpha$  and SAMHD1. Viral restriction by APOBEC3G requires expression of this protein in producer cells (23). Since we produced our viral vectors in 293T cells which don't express APOBEC3G, APOBEC3G cannot be the reason for the block to HIV-1 infection in KG-1 cells. Trim5 $\alpha$  is a pattern recognition receptor that acts on the viral core and prevents reverse transcription to proceed (130, 142). Our results show that reverse transcription processing is not blocked in KG-1 cells suggesting that abnormally acting human Trim5 $\alpha$  is not the reason for the inhibition of HIV-1 and MLV in these cells. Finally, SAMHD1 is a deoxynucleoside triphosphohydrolase which can restrict retroviral infection by depleting the nucleotide pool in non-dividing cells (184, 185, 308). While our qPCR results are partially in line with SAMHD1

restriction, it is highly unlikely that SAMHD1 can have such a substantial effect on infectivity of dividing cells which have high levels of nucleotide pools.

In conclusion, in this study we identify multiple blocks to infection by different viruses and viral vectors in KG-1 cells. Our results indicate that KG-1 cells can be useful tools to identify another component of VSVG binding as well as the mechanism of entry via RD114 glycoprotein. Finally understanding the reason behind the block to retroviral infection in KG-1 cells may lead to identification of a novel restriction factor.

## **Materials and Methods**

### **Reagents and Cell Culture**

293T, Jurkat, KG-1, Cem-A, Molt and Cem-SS cells were obtained from the American Type Culture Collection (ATCC). 293T cells were maintained in Dulbecco's Modified Eagle Medium (Cellgro) supplemented with 10% Fetal Bovine Serum, FBS (Gemini Bioproducts). Rest of the cells were maintained in Iscove's Modified Dulbecco's Medium (ATCC) supplemented with 20% FBS.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; p24 Monoclonal Antibody (183-

H12-5C) from Dr. Bruce Chesebro and Kathy Wehrly, pSV-Ψ-MLV-env- from Dr. Nathaniel Landau.

Goat-anti-mouse-horseradish peroxidase and goat-anti-rabbit-horseradish peroxidase (HRP) secondary antibodies and West Femto enhanced chemiluminescent (ECL) HRP substrate were obtained from Thermo Scientific (Rockford, IL).

Rabbit polyclonal Antibody against gp96 was obtained from GeneTex (Irvine, CA).

Mouse monoclonal antibody against GAPDH was obtained from ABM (Richmond, BC, Canada).

VSVG mouse monoclonal antibody was obtained from KeraFAST.

Dil-LDL was obtained from Kalen Biochemical (Montgomery Village, MD)

VSV-eGFP was a kind gift from Dr. Asit Pattnaik.

Measles-GFP was a kind gift from Stephen Russell.

## **Plasmid Constructs**

Plasmids used for pseudotyped Retrovirus Production:

For HIV-1: CSII-EGFP; an HIV-1 based vector encoding for GFP driven by EF-1a promoter.  $\Delta$ NRF; encodes for gag, pol, rev, tat and vpu of HIV-1. HIG; an NL4-3 based vector described previously was a gift from Dr. Louis Mansky.

pRK5-Nef was cloned after PCR mediated amplification of the nef coding sequence from HIV-1 NL4-3 (obtained from the NIH AIDS reagent program). It was cloned into EcoRI and HindIII sites into the expression vector pRK5 (Addgene).

For MLV: pCLMFG-GFP; an MLV based vector encoding GFP driven by CMV promoter. pCMVgp; encodes for gag and pol of MLV driven by CMV promoter.

Vectors used for retroviral pseudotyping:

pMDg; encodes for vesicular stomatitis virus glycoprotein,

pHCMV-RD114; encodes for RD114 glycoprotein

pSV-A-MLV; Encodes for amphotropic (4070A) MLV envelope

pRK5-10A1; encodes for amphotropic (10A1) MLV envelope

### **Virus Production and Infectivity Assays**

HIV-1 and MLV vectors were generated by transient transfection of three plasmids into 293T cells as described previously (231, 232). For HIV-1 vectors 15 $\mu$ g of CSII EGFP, 10 $\mu$ g of  $\Delta$ NRF and 5 $\mu$ g of the relevant envelope

glycoprotein encoding plasmid were transfected using the method of Chen and Okoyama (233). 72 hours after transfection virus was collected and filtered through a 0.45  $\mu$ M membrane. Filtered virus was concentrated by ultracentrifugation (100,000  $\times$  g, 2 hours at 4°C). Viral pellet was resuspended in Phosphate buffer saline (PBS) and aliquots were stored at -80°C. Viral titers were determined by infecting  $1 \times 10^5$  Jurkat cells with 10 fold dilutions of the viral preparation. 72 hours after the infection EGFP expression was quantified by flow cytometry on a Becton-Dickinson FACScalibur. Same procedure was followed for production of MLV vectors using following plasmids; 15 $\mu$ g of pCLMFG-GFP, 10 $\mu$ g of pCMVgp and 5 $\mu$ g of the relevant envelope glycoprotein.

### **Reverse transcription products qPCR assay**

$1 \times 10^5$  cells were plated into 6 well dishes and infected at an MOI of 1. Cells were kept at 4°C for 1 hour before and after the addition of virus to synchronize the infections. Controls consisted of uninfected cells or cells infected with heat inactivated virus for 36 hours. Cells were harvested and washed with PBS at different time points; 8 hours for first jump products, 48 hours for 2LTR circles and 6 weeks post infection for full product. Cell lysates were prepared by resuspending the cell pellet in lysis buffer (Tris pH 8.0, 25 mM EDTA pH 8.0, 100 mM NaCl, 1% Triton X-100, and 2 mg/ml proteinase K) and incubating at 55°C overnight. Proteinase K was inactivated by treating the lysate at 95°C for 15

minutes. Lysates were used directly for qPCR analysis. Following primers were used for qPCR [57]: for first jump products; U31 - GGA TCT ACC ACA CAC AAG GC, U32 – GGG TGT AAC AAG CTG GTG TTC. For 2LTR circles: MH535 – AAC TAG GGA ACC CAC TGC TTA AG, MH536 – TCC ACA GAT CAA GGA TAT CTT GTC. For Full product: LTR9 - GCC TCA ATA AAG CTT GCC TTG, AA55 - CTG CTA GAG ATT TTC CAC ACT GAC  $\beta$ -actin- ATC ATG TTT GAG ACC TTC AA, 3'  $\beta$ -actin- AGA TGG GCA CAG TGT GGG T. QPCR reactions using SYBR green were performed using Eppendorf real plex master cycler ep and BioRad SYBR SuperMix according to manufacturer's protocol. Cycling conditions used were 95°C for 2 min, followed by 40 cycles of 95°C 30s, 58°C 30s, and 72°C 30s, and a final extension of 5 minutes at 72°C for all PCR products. Cycle threshold value was used to normalize the DNA amounts for the Jurkat cells. Fold difference was calculated using the Delta Ct method. The melt curve as well as analysis of the PCR products by agarose gel electrophoresis confirmed the presence of one product at the expected size (data not shown). DNA input was controlled by qPCR amplification of a fragment of the  $\beta$ -actin gene.

### **Viral entry and binding assays and immunoblotting**

For VSVG binding and entry assays  $1 \times 10^6$  KG-1 and Jurkat cells were incubated with HIV-VSVG (MOI = 5) at 4°C for 1 hour. Infection was started by

moving the cells into 37°C. Cells were collected at different time points, washed 10 times with cold PBS. For the entry assay cells were treated with 0.05% Trypsin-EDTA (Life Technologies) for 15 minutes at 37°C to remove surface bound virions and then washed 10 times again with cold PBS. Washed cells were lysed with lysis buffer (20 mM Tris-HCl [pH 8.0], 1 mM CaCl<sub>2</sub>, 150 mM NaCl, 1% Triton) and proteins were separated with SDS-PAGE on 12% polyacrylamide gel. Presence of virus and cell proteins were detected with immunoblotting using the antibodies indicated. Same procedure was followed for RD114 mediated HIV-1 binding and entry assays. Cells were collected at 2 hours post infection and either treated with Trypsin-EDTA (for entry) or just washed (for binding).

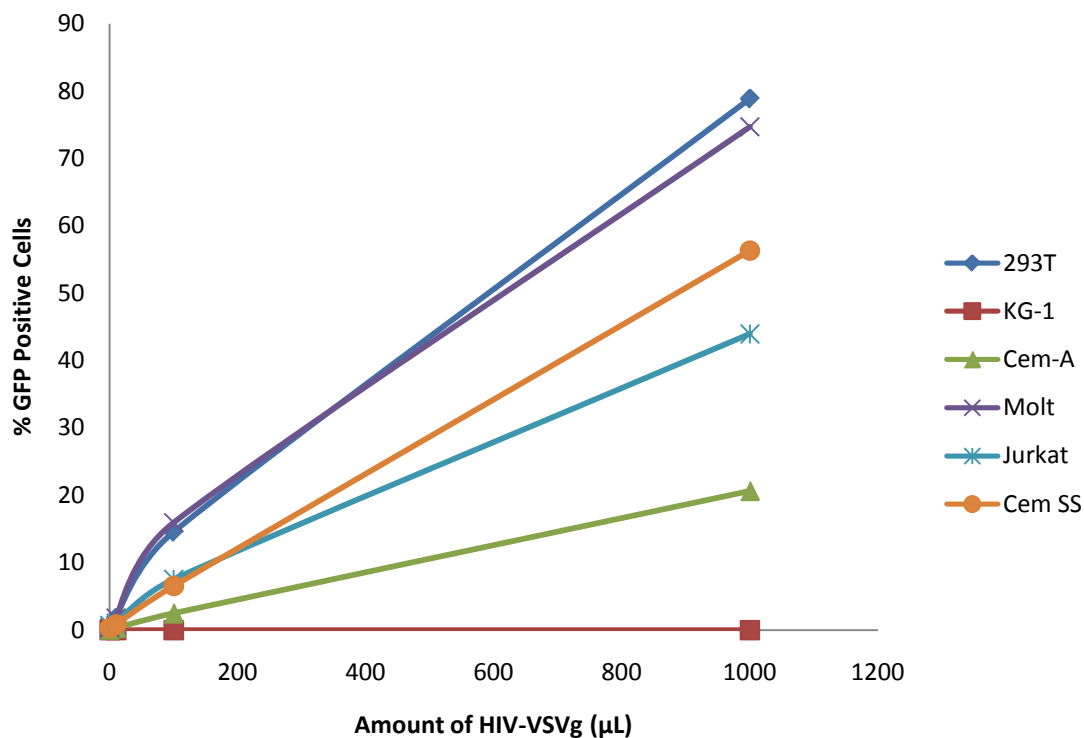
Flow cytometry based VSVG binding assay was performed by first incubating  $1 \times 10^6$  KG-1 and Jurkat cells with HIV-VSVG (MOI=5) at 4°C for 2 hours. Following extensive washes with cold PBS samples were incubated with anti-VSVG antibody for 1 hour at 4°C. Unbound antibody was washed with cold PBS and secondary antibody with conjugated Alexa488 was added to the cells and incubated at 4°C for 1 hour. Following several washes with cold PBS fluorescent cells were detected using Becton-Dickinson FACScalibur.

For detection of gp96 expression,  $5 \times 10^5$  KG-1 and Jurkat cells were lysed with lysis buffer (20 mM Tris-HCl [pH 8.0], 1 mM CaCl<sub>2</sub>, 150 mM NaCl, 1% Triton), proteins were separated with 12% SDS-PAGE and gp96 was detected with immunoblotting.

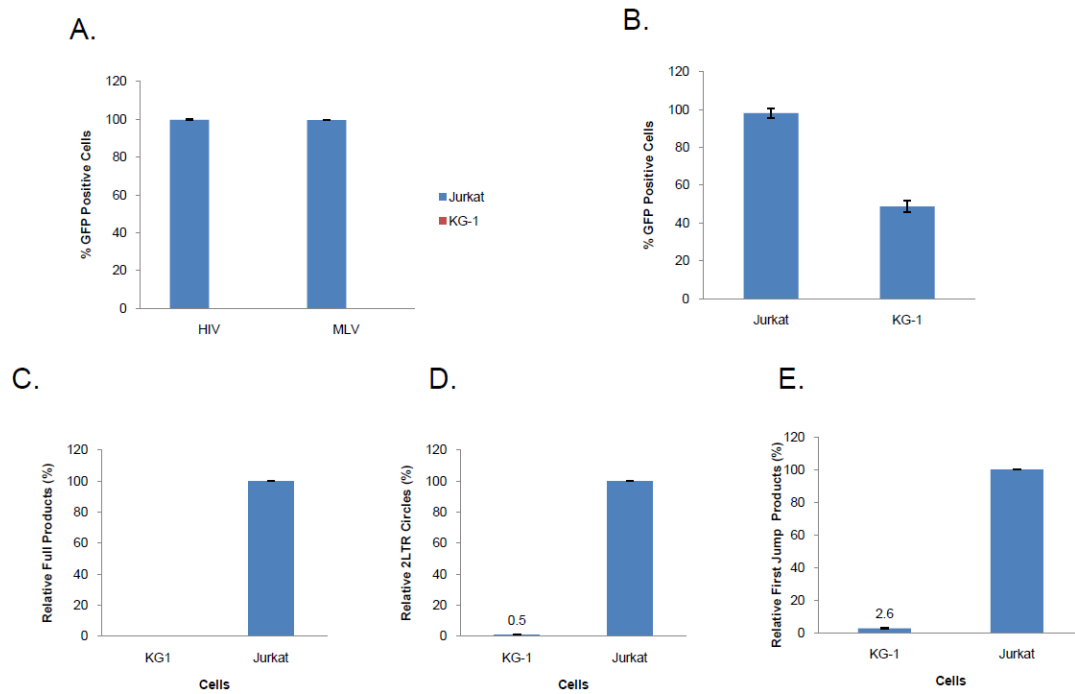


**LDL uptake assay**

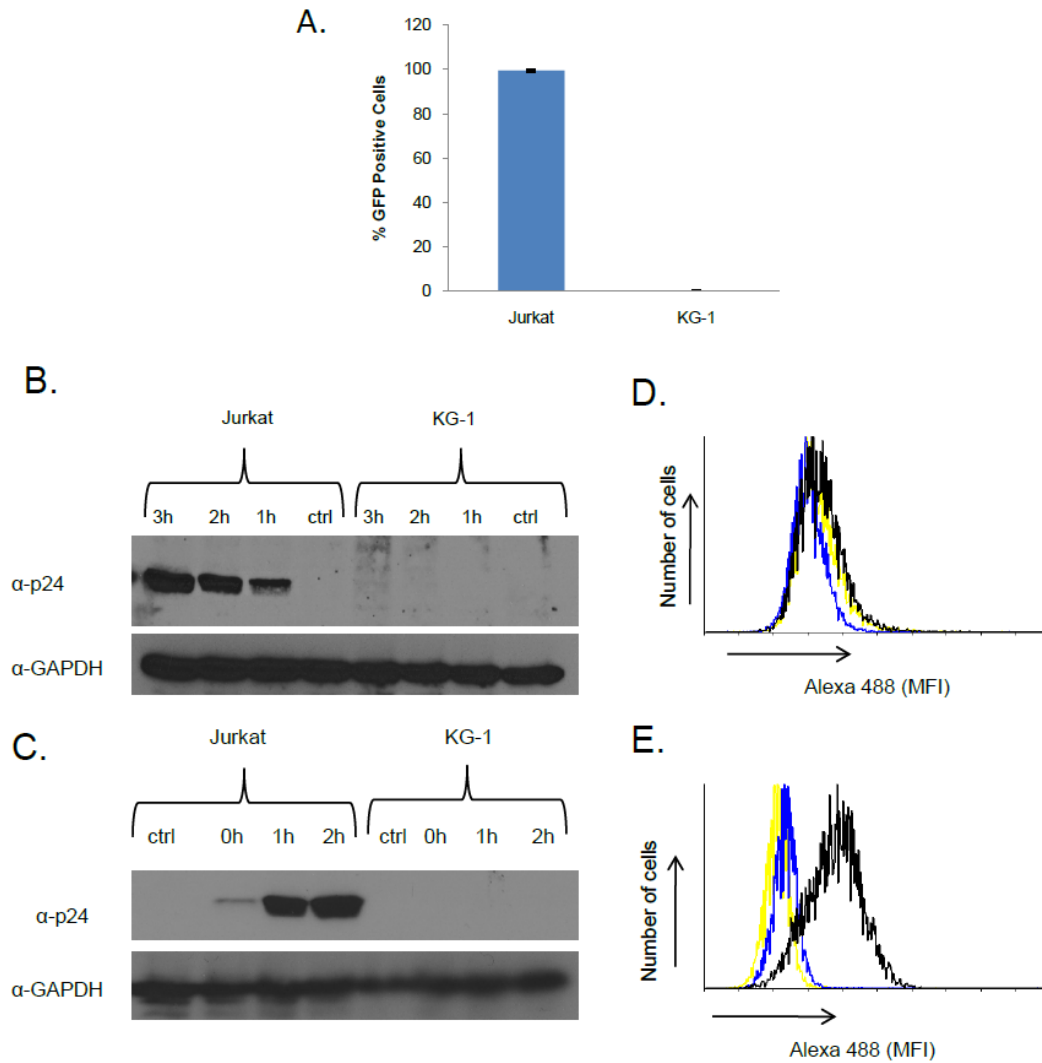
1 x 10<sup>6</sup> KG-1 and Jurkat cells were plated into 6 well plates in serum free media. 3 µg/mL Dil-LDL was added and the cells were incubated for 4 hours. The cells were then washed 3 times with PBS and treated with 0.05% Trypsin-EDTA at 37°C for 15 minutes to remove surface bound Dil-LDL. After several washes with PBS, fluorescence of cells was scored using Becton-Dickinson FACScalibur.



**Figure 4-1. Infectivity of different human cell lines with HIV-VSVG.** Several human cell lines were transduced with different amounts of HIV-VSVG that was produced in 293T cells. Percent of GFP positive cells were determined using flow cytometry 3 days post infection.



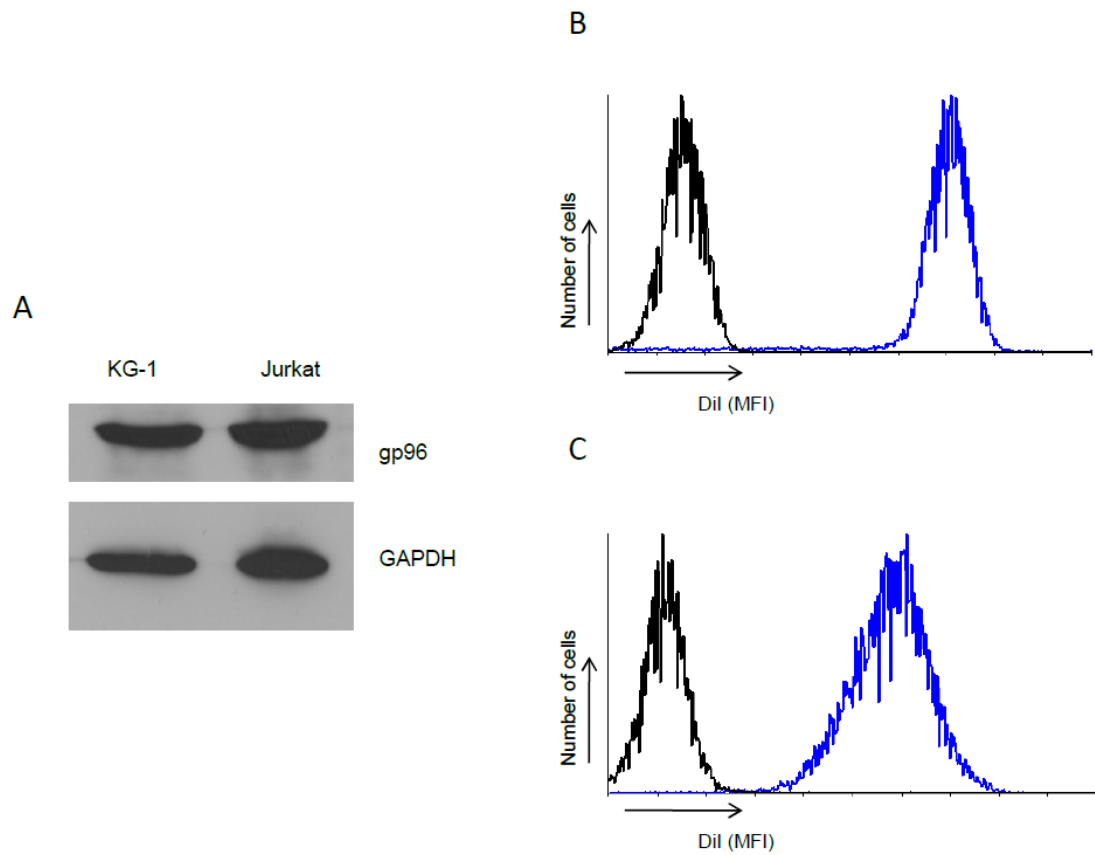
**Figure 4-2. KG-1 cells cannot be infected with VSVG pseudotyped retroviruses.** **A.** KG-1 and Jurkat cells were infected with HIV-VSVG and MLV-VSVG at an MOI of 10. Percent of GFP positive cells were determined using flow cytometry 3 days post infection. **B.** KG-1 and Jurkat cells were infected with Measles virus encoding GFP at an MOI of 3. Percent of GFP positive cells were determined using flow cytometry 48 hours post infection. **C, D and E.** KG-1 and Jurkat cells were infected with HIV-VSVG at an MOI of 1. Viral cDNA products were quantified using qPCR for integrated provirus at 6 weeks post infection (**C**), for 2LTR circles at 48 hours post infection (**D**) and for first jump products 8 hours post infection (**E**).



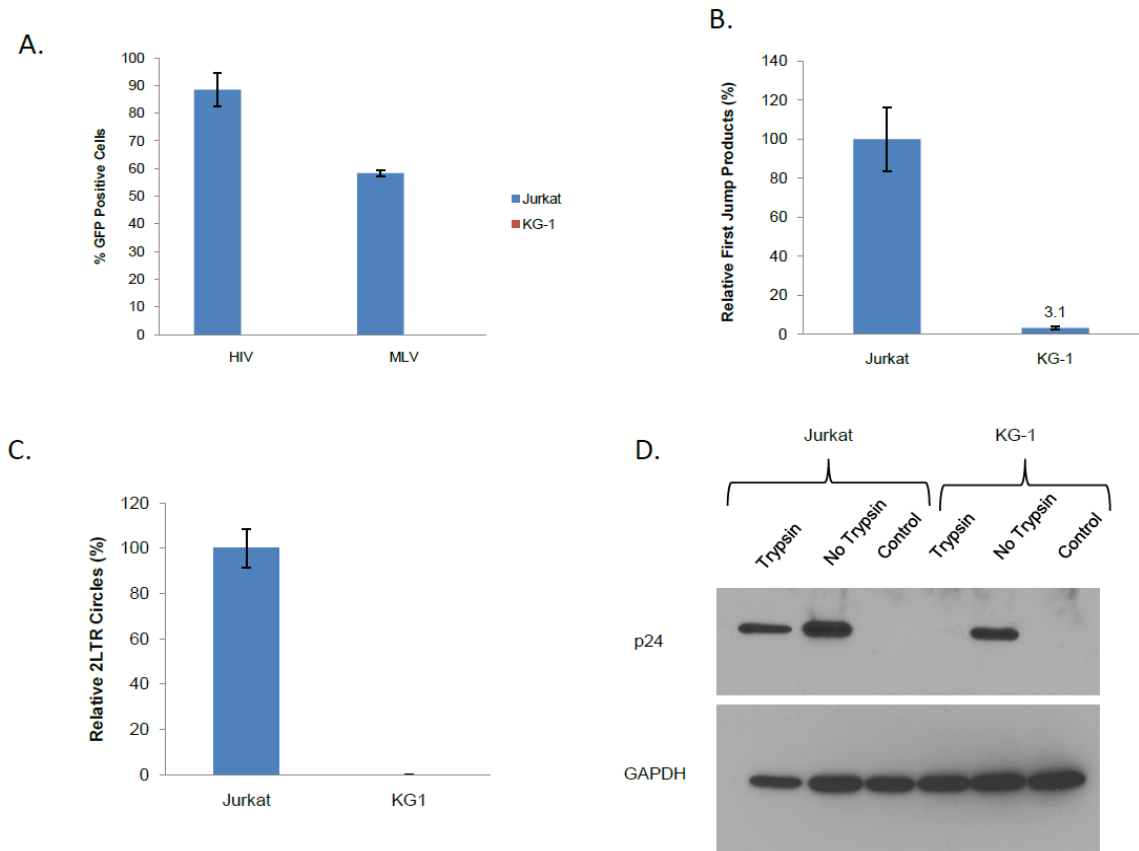
**Figure 4-3. VSVG binding is impaired in KG-1 cells.** **A.** KG-1 and Jurkat cells were infected with VSV-eGFP at an MOI of 50. Percentage of GFP positive cells were determined at 24 hours post infection using flow cytometry. **B.** KG-1 and Jurkat cells were infected with HIV-VSVG at an MOI of 5. Cells were collected at

indicated times, washed, treated with Trypsin-EDTA and the lysates were used for immunoblotting with the indicated antibodies. Ctrl indicates non-infected cells.

**C.** KG-1 and Jurkat cells were infected with HIV-VSVG at an MOI of 5. Cells were collected at indicated times, washed and the lysates were used for immunoblotting with the indicated antibodies. **D and E.** KG-1 (**D**) and Jurkat (**E**) cells were incubated HIV-VSVG at an MOI of 5 at 4°C for 2 hours. Cells were collected, washed and incubated with an antibody against VSVG and a secondary antibody with Alexa488 conjugated to it. Surface bound virions were detected with flow cytometry. Yellow line: Non - infected cells. Blue line: Non - infected cells incubated with antibodies. Black line: Infected cells incubated with antibodies.



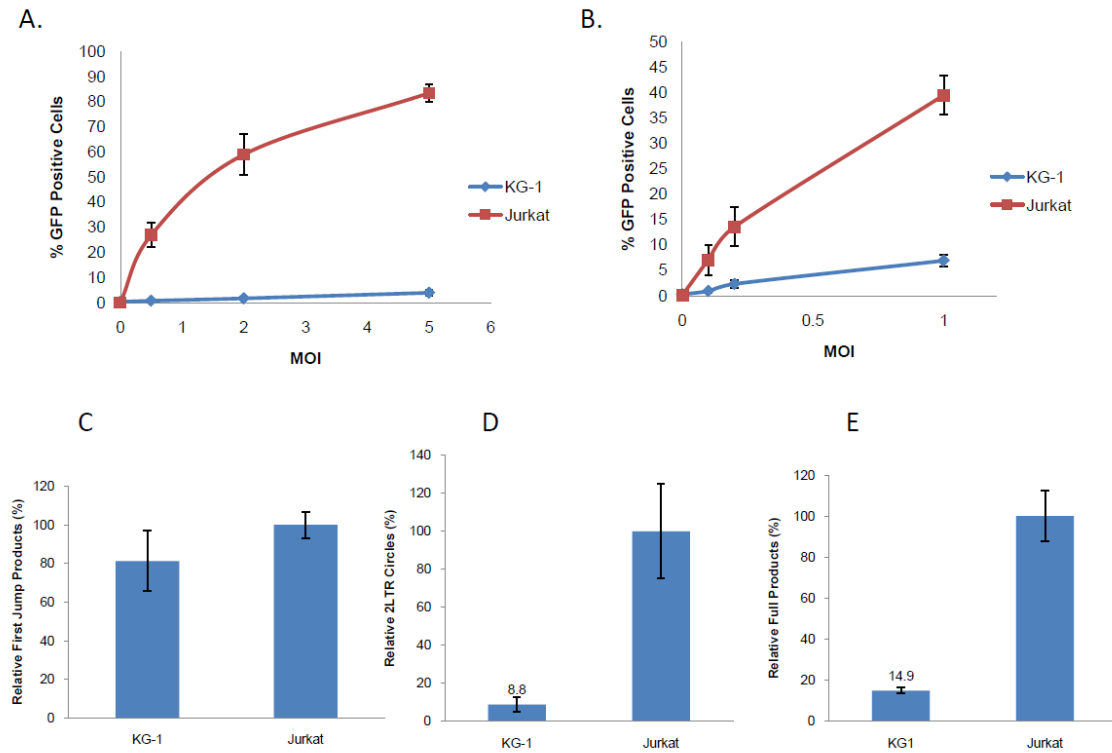
**Figure 4-4. KG-1 cells express gp96 and can functionally take up LDL. A.** Immunoblots of KG-1 and Jurkat cell lysates using gp96 and GAPDH antibodies. **B and C.** KG-1 (**C**) and Jurkat (**B**) cells were incubated with DiI-LDL for 4 hours and fluorescent cells were detected using flow cytometry.



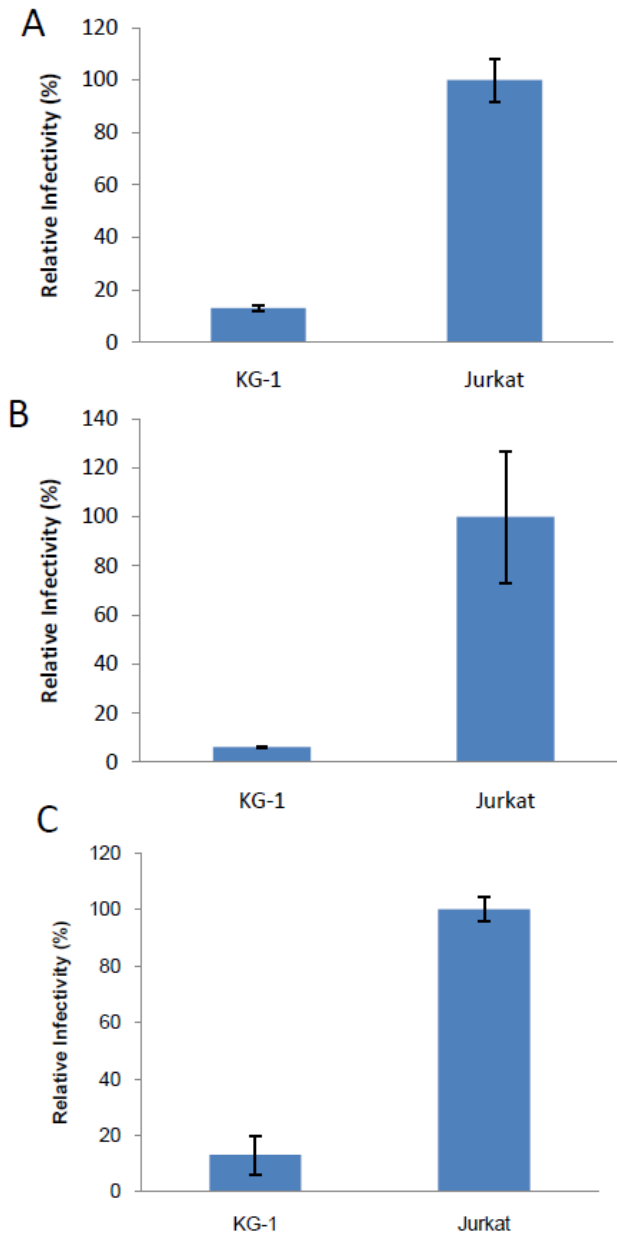
**Figure 4-5. RD114 mediated entry deficiency in KG-1 cells.** **A.** Jurkat and KG-1 cells were infected with RD114 pseudotyped HIV-1 (MOI = 5) and MLV (MOI = 1) encoding GFP. Percentage of GFP positive cells were determined using flow cytometry 3 days post infection. **B and C.** KG-1 and Jurkat cells were infected with RD114 pseudotyped HIV-1 at an MOI of 1. Viral cDNA products were quantified using qPCR for first jump products 8 hours post infection (**B**). for 2LTR circles at 48 hours post infection (**C**). **D.** KG-1 and Jurkat cells were infected with RD114 pseudotyped HIV-1 at an MOI of 5. Cells were collected at 2 hours,

washed, treated with Trypsin-EDTA or not and the lysates were used for immunoblotting with the indicated antibodies. Control indicates non-infected cells.





**Figure 4-6. KG-1 cells are refractory to infection with retroviruses pseudotyped with amphotropic envelope** **A.** KG-1 and Jurkat cells were infected with 4070A pseudotyped MLV-GFP at different MOIs using spinoculation. Percentage of GFP positive cells were determined 3 days post infection. **B.** KG-1 and Jurkat cells were infected with 4070A pseudotyped HIV-GFP at different MOIs using spinoculation. Percentage of GFP positive cells were determined 3 days post infection. **C, D and E.** KG-1 and Jurkat cells were infected with 4070A pseudotyped HIV-1 at an MOI of 1. Viral cDNA products were quantified using qPCR for first jump products 8 hours post infection (**C**), for 2LTR circles at 48 hours post infection (**D**) and for integrated provirus at 6 weeks post infection (**E**).



**Figure 4-7. KG-1 cells can maintain integrated provirus and HIV-1 accessory factors don't rescue the block to infection. A.** KG-1 and Jurkat cells were infected with 4070A pseudotyped HIV-GFP at an MOI of 0.5 using spinoculation. Percentage of GFP positive cells were determined 4 weeks post

infection. **B.** KG-1 and Jurkat cells were infected with 4070A pseudotyped MLV-GFP at an MOI of 1 using spinoculation. Percentage of GFP positive cells were determined 4 weeks post infection. **C.** KG-1 and Jurkat cells were infected with 4070A pseudotyped HIV-GFP containing all the accessory factors (HIG). Percentage of GFP positive cells were determined 3 days post infection.

## **Chapter 5**

### **Conclusions and Future Experiments**

The goal of this dissertation is to increase our understanding of the interaction between HIV-1 and the host cell. I utilized two strategies to achieve this goal: 1. Investigating the role of a ubiquitous cellular pathway (the N-end rule) in HIV-1 replication cycle. 2. Characterizing a human cell line resistant to HIV-1 and HIV-1 based vectors

N-end rule pathway had previously been implicated in the HIV-1 life cycle both directly through RNAi screens and indirectly via the observation that integrase can be a substrate for this pathway when expressed ectopically in cells (49, 203, 205). Using this information I investigated the effect of the N-end rule pathway on the integrase protein of HIV-1 and MLV during the early phase of viral life cycle. As presented in Chapter 2, the results obtained using cells that are genetically impaired in the N-end rule pathway and viruses that have mutations at the integrase N-terminal residue indicate that integrase is not a direct target of the N-end rule pathway during HIV-1 and MLV life cycles. Integrase or any other viral proteins that are part of the GagPol polyprotein cannot be a substrate of N-end rule during the late phase of the viral life cycle since these substrates are only revealed after maturation. Therefore, the N-end rule can only directly act on these proteins when they are delivered into the cell via the viral particle.

Interestingly depletion of E3 ligases of the N-end rule pathway impacted HIV-1 and MLV infections differently. Ubiquitous nature of this pathway combined with the results we obtained on integrase suggests that this difference is caused by the effect of N-end rule impairment on the stability of one or more host proteins. Because only a limited number of targets of N-end rule pathway in the mammalian cells have been identified, the reason for the observed decrease in HIV-1 infectivity may not be trivial to find. However there are other potential avenues for further investigating the impact of the N-end rule pathway on HIV-1 life cycle.

One such experiment would be to knockout UBR1, 2 and 4 in human cells. With the development of efficient site specific targeting technologies in recent years, it is now possible to mutate a specific gene or even a specific region in a gene with relative ease in cell culture (309-311). Experiments presented in chapter 2 relied on mouse embryonic fibroblasts with gene knockouts. It is important to test whether depleting the UBR proteins of the N-end rule will have the same effect on HIV-1 or MLV infectivity in human cells. In addition UBR4 depletion was achieved through RNAi, hence reduced infection that we observed could be enhanced when this protein is completely absent in the cells. Moreover, UBR5 was previously identified as an HIV-1 host cell factor in a genomewide RNAi screen (49). In this study we didn't test the role of UBR5 in N-end rule and integrase due to the fact that integrase stability was previously shown to be

impacted in cells depleted of UBR1,2 and 4. Given the observed redundancy between UBR proteins, knockout studies may reveal useful information about the effect of UBR5 on HIV-1 infection.

As mentioned above, the N-end rule cannot have a direct effect on viral proteins with destabilizing N-terminal residues in the late phase of the viral replication. However, cellular proteins that are substrates of the N-end rule pathway may be involved during the late phase. This can be tested by transfecting human cells engineered for UBR knockouts with the three plasmid system described in the previous chapters for HIV-1 production. Comparison of production of Gag, GagPol as well as the amount of virus released from the cells with differential N-end rule pathway capabilities will reveal whether the N-end rule pathway affects the late phase of retroviral life cycle.

While studying the potential impact of the N-end rule pathway on HIV-1 RNase H, we discovered an intriguing phenomenon; changing the N-terminal residue of RNase H into a structurally different amino acid than the conserved residue led to the absence of RT in the virus particle. Our results, as presented in chapter 3, indicate that this is caused by the degradation of RT in the virus particle after its release from the GagPol polyprotein following virus budding. Interestingly we found a defect in heterodimerization of RT subunits in one of the RNase H

mutants. This is the first time a residue in RNase H has been shown to affect RT subunit dimerization. At this point it is unknown whether this defect in subunit binding is the cause of intravirion RT degradation. There is evidence from previous studies of a decrease in the amount of RT in virus particles when heterodimerization impaired mutants were used (268, 269). While this decrease doesn't match the substantial degradation we observed for all the unstable mutants generated in this dissertation, it is possible that the N-terminal RNase H residue is more critical for subunit binding than the previously tested residues. Therefore further experimentation is needed to better understand the role of this residue in RT subunit interactions. A useful starting point would be to test the heterodimerization of RT subunits in the other RT unstable RNase H mutants we generated. Some of the mutants showed residual levels of RT in the virus particle. Similarly a lessened affect may be observed in subunit binding impairment. In addition, the rest of the naturally occurring amino acids can be tested for both intravirion RT stability and heterodimerization. Interestingly the RT inhibitor antiretroviral drug efavirenz has been shown to enhance the dimerization of RT subunits and rescue a defect in RT subunit dimerization in some RT mutants (266, 312). Testing the effect of efavirenz in the RNase H N-terminal mutants may reveal clues about the mechanism of the heterodimerization defect.



Results presented in chapter 3 suggest that one or more cellular proteases may be responsible for the degradation of intravirion RT with N-terminal RNase H mutations. Unfortunately we were unable to test this stringently in virus particles because the RNase H proline mutant had a defect in dimerization of RT subunits as mentioned above. Since our results indicate that RT needs to be released from the fusion protein to be subjected to degradation in the virus particle, testing of RNase H mutant RT degradation in protease inactive virions presents a challenge. It is possible that N-terminal mutants that contain other amino acids that we haven't tested are still subject to the same degradation while retaining the subunit binding ability.

One of the RNase H N-terminal mutants (Methionine) showed residual infectivity in Jurkat cells. Using replication competent HIV-1 it may be possible to isolate viruses that rescue the partial RT degradation observed in the methionine mutant. Sequencing of these mutants may reveal important sites for RT stability including possible sites that may rescue the dimerization defect we observed for the proline RNase H mutant. If any of these mutations can rescue the RT subunit dimerization without changing RT stability inside viral particles, it can be used to test whether viral protease is responsible for this degradation or not.

Whether the degradation of RT in RNase H mutants is mediated by a cellular protease or the viral protease, it represents an interesting drug target in RT. Targeting of protease cleavage sites is not a new idea. In fact an experimental antiretroviral called bevirimat was shown to inhibit the maturation process of HIV-1 Gag by binding to the protease cleavage site between capsid and p1 spacer protein (313). Even though this drug wasn't specifically designed to bind a protease cleavage site, it shows the viability of this type of targeting.

In the final part of this dissertation, I have examined the resistance to retroviral infection in a human myeloid cell line. Remarkably KG-1 cells turned out to be defective in binding to VSVG. To my knowledge this represents the first human cell line that is incapable of supporting entry by VSV. Future experiments can elucidate the reason behind this binding defect. My preliminary observation suggests that this defect may be dominant, indicating a host protein may be preventing the VSVG receptor to be synthesized or to reach the cell surface. This preliminary analysis needs to be confirmed and if heterokaryons reveal that the block is indeed dominant, cDNA library transfer can be used to identify the gene responsible. If the opposite is true, it may be possible to transduce KG-1 cells with a cDNA library from a permissive cell type using a retroviral vector with amphotropic envelope. Low levels of infectivity I observed with these vectors may be sufficient to identify the missing host factor. A similar strategy can be used to

find the identity of the host factor responsible for causing the restriction of retroviral infection and RD114 entry defect.

Resistance to amphotropic envelope pseudotyped retroviruses observed in KG-1 cells seems to be caused by a block at late reverse transcription or nuclear import. Even though I observed similar resistance to viruses expressing all HIV-1 accessory factors, it is possible that another retroviral accessory factor, Vpx, may relieve this inhibition. This can be tested by infecting KG-1 cells with HIV-2 or by transducing the cells with virus like particles containing Vpx before infecting them with HIV-1 or MLV (183-185, 314, 315). A recently identified target of antagonism by Vpx, SAMHD1, is unlikely to be acting on these cells since as dividing cells KG-1 cells require high levels of dNTPs. Nevertheless it is possible that an unidentified host factor that is counteracted by Vpx may be responsible for the resistance to retroviral infection in these cells.

In conclusion, the studies presented in this dissertation have increased our understanding of the interaction of HIV-1 with host cell factors. It was previously hypothesized that HIV-1 integrase may be a target for the N-end rule pathway. I have accumulated substantial evidence to contradict this claim using N-end rule deficient cells and Integrase N-terminal mutants of HIV-1 and MLV. Moreover in an effort to understand the role of RNase H N-terminal residue in HIV-1 infectivity

I have discovered that this residue is not only critical for viral infectivity, it is also important for RT stability in the virus particle and dimerization of RT subunits. In addition to the studies of N-end rule and N-terminal residues of retroviral proteins, I have also identified the first human cell line that is resistant to binding by VSVG. This observation may be used to identify critical components of VSVG mediated entry into cells. Moreover my results indicate that this cell line, KG-1, is refractory to retroviral infection due to a block in late reverse transcription or nuclear import. Further studies of KG-1 cells may reveal the identity of a novel restriction factor or a critical cofactor in HIV-1 infection.

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